Final scientific report on the decision support project

"Investigation into the presence of 3-MCPD esters and related compounds in foods."

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1) Project objectives and remit

Chlorinated propanediols such as 3-chloropropane-1,2-diol (3-monochloropropane-1,2-diol, 3-MCPD) and the isomeric compound 2-chloropropane-1,3-diol (2-monochloropropane-1,3-diol, 2-MCPD) are thermally induced process-related contaminants, which may be present in a variety of foods both in their free form and esterified with fatty acids as "bound MCPD". While the toxicological relevance of free or bound 2-MCPD is not proven due to a lack of scientific data, free 3-MCPD was shown to be carcinogenic in *in-vivo* studies with rats. The glycidyl ester ("bound glycidol") substance class, which is structurally similar to fatty acid-bound MCPD and its occurrence associated with this compound, is classified as a probable human carcinogen. There is now sufficient evidence to show that the parent compounds 2-MCPD, 3-MCPD and glycidol are substantially or fully released from their fatty acid esters during digestion in the intestinal tract [Barocelli et al.: 2011, Buhrke et al.: 2011, Abraham et al.: 2012, Appel et al.: 2013, Buhrke et al.: 2015].

The results of a series of publications suggest that in the main, the temperatures of more than 200 °C normally used when deodorising oils and fats, cause the formation of bound MCPD from natural fat components and various chloride sources. Fatty acid-bound glycidol can also be formed under the same conditions [Weißhaar: 2008a, Weißhaar et al.: 2010, Hrncirik et al.: 2011, Ermacora et al.: 2014]. Furthermore, there are indications and evidence to show that MCPD and glycidyl derivatives can also be formed under certain conditions when preparing food (smoking, roasting, baking, frying etc.) [Hamlet et al.: 2002, Weißhaar: 2011]. It is also not possible to exclude a migration of free 3-MCPD from packaging materials into the food [Becalski et al.: 2015] or the release of MCPD from its bound form as a result of physicochemical or enzymatic processes during further food processing or storage [Hamlet et al.: 2004 a,b]. Consequently, it can be assumed that refined oils and fats, as well as food that contains refined oils and fats, contribute greatly to consumers being exposed to MCPD and glycidol. The European Commission has therefore advised all Member States to monitor the presence of free 2- and 3-MCPD, bound 2- and 3-MCPD, and bound glycidol in foods [EU: 2014].

However, current exposure data are not readily available because the prevalence of MCPD and glycidyl derivatives in representative food groups has not been adequately investigated. The reasons for this are that the necessary sophisticated and complex trace analytics to detect all five of the specified contaminants is only found in a few laboratories. In addition, the focus of studies to date has been more on refined edible oils and fats, whereas compound foods that

contain oils and fats are less researched, partly due to a lack of officially validated analytical methods. In 2013, the European Food Safety Authority (EFSA) published a preliminary exposure assessment based on the presence of 3-MCPD in foods [EFSA: 2013]. This was followed in 2016 by a scientific opinion by the EFSA Panel on Contaminants in the Food Chain (CONTAM) on the risks of the occurrence of free and bound 2- and 3-MCPD, and glycidyl esters, which was based on a large quantity of data produced from several years of studies. However, these data came from a variety of different analytical methods, the application and comparability of which had only been tested to a limited extent. The largest datasets were based on oils, fats and margarines, and were provided by economically dependent organisations. The number of compound foods studied was therefore low and can only be considered to be a basis for an initial rough estimate. [EFSA: 2016]. These shortcomings gave rise to the objective of this project – to determine the content of free 2- and 3-MCPD and separately the content of bound 2-MCPD, 3-MCPD and glycidol for a series of relevant food groups, each in a meaningful number of samples. This will be achieved in such a way as to obtain comparable, representative results across batches to significantly support a current exposure estimation. In particular, infant formula, edible oils, margarine, frying fats, French fries, croissants, doughnuts, spreads and Asian dishes with dried noodles to a total of 1050 individual samples from the German food trade, was to be purchased and quantitatively analysed for the specified contaminants.

2) Project planning and schedule

The project planning is based on the continuous purchase of all samples by SGS Germany GmbH without differentiating according to food groups. Purchases were be made in consultation with the Federal Institute for Risk Assessment (BfR) regarding the specific sampling plan via online portals or on-site samplers. In principle, the intention was to include a range of supermarkets, self-service stores, discount stores, and drugstores in the sampling and to purchase takeaway food samples in bakeries, kiosks and food stalls. Food was to be purchased as random or representative samples, in other words so that the product selection within the defined food products was random but so that the selected products were numerically proportional to the market shares of the food retail companies. Following the documented purchase, all samples were to be promptly sent to the laboratory site in Hamburg-Bergedorf, Germany and here, in accordance with the Federal Office for Agriculture and Food (BLE) and before analytical testing, all the relevant product information (product name, batch, date of purchase, place of purchase, anomalies) should be recorded. To achieve the tight time framework of submitting the analysis results with six months of the award of contract, the intention was to analyse the samples for contaminants continuously and without extensive pooling according to food groups.

To analyse all the foods included in the project scope, analytical methods were proposed that were based on the officially validated AOCS Method Cd 29b-13 (SGS "3-in-1") for the determination of bound 2-MCPD, 3-MCPD and glycidol for edible oils and fats. This required adaptation of the analytics to record the free analytes separately and to achieve the low limits of quantification required within the project scope for the bound analytes. For the compound foods for which there were no available officially validated methods to determine all three bound analytes at the time the project was completed, the intention was to use the so-called SGS "5-in-2" method. This is a procedure based on the AOCS Cd 29b-13 method and validated in-house, with which practically all solid compound foods can be analysed for the free and bound analytes. To generate the most meaningful data on the presence of the specified contaminants in various foods, it was planned to keep the limits of quantification as low as possible; in this case, at least per 5 µg/kg product for the free analytes and at least per 25 µg/kg product for the bound analytes. Finally, an interface to the data reporting portal should be used for data transmission.

The purchase of samples and all the laboratory work was to be performed under accredited conditions and correspond to the highest possible standards in terms of the technical standard,

quality assurance, data traceability and professional support. From a technical perspective, a modern gas chromatograph with a mass-selective detector (GC-MSD) was also provided by Agilent Technologies for the technology part. The intention was to avoid measuring any other routine samples on the system during the project phase, with the exception of quality assurance measures, to prevent any interference effects by unknown matrices and to perform as little maintenance work as possible. The practical work i.e. purchasing and sample preparation, was to be assigned to trained lab personnel without referencing specific people, whereas the project manager was responsible for evaluating the quality assurance measures, analysing the raw data, checking the plausibility of the measurements and the final report.

The project could largely be completed in line with the schedule. Following the award of contract on 28 December 2015, there was a preliminary discussion in Bonn on 29 January 2016. A detailed sampling plan was gradually clarified and established by the BfR which meant clearly defined product groups could start being purchased in February 2016. Outstanding questions for other foods could be resolved and sampling begun at a later date. During the project, adjustments were made for several food groups regarding the product selection and the extent of sampling. The reasons for this were, for instance, a limited availability of certain foods or the evaluation of preliminary analytical results that were regularly provided and explained to the BfR as interim advance information. As a result of the relatively late availability of the full sampling plan and the sensible adjustments to the food purchases at an advanced stage of the project, the originally notified time frame that anticipated submission of all the measurements 6 months after signing the contract, was extended to 14. October 2016. The analytical part and data transmission proceeded without interruption and complied in terms of the methodology with the achieved limits of quantification and the analytical significance defined in the planning specifications.

3) Scientific and technical state of the art that provided the basis for further work.

3.1) Toxicity & metabolism

There are insufficient studies on 2-MCPD to reliably assess its toxicity. Toxic effects on muscles, the heart, liver and kidneys in rats following oral administration of high doses of up to 30 mg 2-MCPD/kg bodyweight (bw) for 28 days were derived from unpublished studies [Andres et al.: 2013]. In comparison, 3-MCPD is relatively well researched from a toxicological perspective. Data from a series of *in-vitro* and *in-vivo* studies are available that prove, for instance, non-genotoxic carcinogenicity [Barocelli et al.: 2011, Bakhiya et al.:

2011]. The International Agency for Research on Cancer (IARC) assesses 3-MCPD as possibly carcinogenic to humans (category 2B) [IARC: 2013]. Glycidol, which is also used as a technical chemical, was previously classified by the IARC as a genotoxic carcinogen that is probably carcinogenic to humans (category 2A) [IARC: 2000]. In recent years, results from animal studies have been published in which a substantial or full release of contaminants from the esterified form was shown with a high degree of certainty for 3-MCPD esters and glycidyl esters. In addition to the paper by the University of Parma cited above "Comparison between 3-MCPD and its palmitic esters in a 90-day toxicological study" [Barocelli et al.: 2012], these studies also included the previous projects "*EH-Forschungsvorhaben 2808HS013: Tierstudie zur Untersuchung der Bioverfügbarkeit und Metabolisierung von 3-MCPD-Estern*" [Decision support project 2808HS013: animal study to investigate the bioavailability and metabolism of 3-MCPD esters] [Abraham et al.: 2013] and "*EH-Forschungsvorhaben 2809HS013: Tierstudie zur Untersuchung der Bioverfügbarkeit und Metabolisierung von Glycidyl-Fettsäureestern*" [Decision support project 2808HS013: animal study to investigate the bioavailability and metabolism of glycidyl fatty acid esters] [Appel et al.: 2013].

According to the level of scientific understanding of its presence and toxicology, a tolerable daily intake (TDI) of 2 µg/kg bw was specified for free 3-MCPD in 2001 by the EU Expert Committee (SCF), and in subsequent years this was confirmed by various institutions (WHO, EFSA, BfR) [SCF: 2001, BfR: 2012]. This was followed by EU quantitative limits of 20 µg/kg in soy sauces and hydrolysed plant protein (based on a dry matter of 40 %) [EU: 2001], and 100 µg/kg in glycerin, when this is used as a food additive [EU: 2012]. Due to the initially uncertain data on to what extent 3-MCPD is released from the esterified form during digestion, the EFSA and the BfR have supported a risk assessment based on the worst-case scenario of full release and consequently estimated the TDI value at 2 µg/kg for the bound 3-MCPD as well [BfR: 2012]. In the scientific opinion published by the EFSA in May 2016 on the exposure and risks of free and bound 2- and 3-MCPD and bound glycidol in foods, a group TDI value for free and bound 3-MCPD of 0.8 µg/kg bw was derived using a new calculation basis for toxicity. This derivation is also based on the estimation that the toxicity of 3-MCPD fatty acid esters in its molar equivalent is equated with that of the free compound [EFSA: 2016]. In the case of suspected genotoxic substances such as glycidol, its content in food should be kept *As Low As Reasonably Achievable* – the ALARA principle. The derivation of a threshold or TDI value is not applicable in this case. Assessment is possible using the *Margin of Exposure* (MoE). The BfR also published an assessment for glycidyl ester

back in 2009 in which the MoE may fall below 10000 if the daily intake of glycidol equivalents exceeds a value of 0.406 µg/kg bodyweight. For infants that are not breastfed and who ingest large quantities of fat proportional to their bodyweight from commercial infant formula, this approach means that even contents of 67 µg/kg fat in the foodstuff can fall below the MoE [BfR: 2009]. If you assume that infant formula contains an average fat quantity of about 25 %, on this basis these products should not contain more than approx. 17 µg/kg of bound glycidol.

3.2) Presence in foods

The development of monochlorinated and dichlorinated propanols as process contaminants in food production, particularly in the manufacture of protein hydrolysates by means of hydrolysis with hydrochloric acid, was described back in the late 1970s [Velisek et al.: 1979, Velisek et al.: 1980, Davidek et al.: 1982]. It was later found that free 3-MCPD, and to a lesser extent also free 2-MCPD, could not only occur in vegetable protein hydrolysates such as soy sauce and relishes, they could also be produced in many heating processes such as roasting, smoking or grilling foods [Crews et al.: 2001, Hamlet et al.: 2002, Kuntzer et al.: 2006, Weißhaar: 2011]. As early as the 1980s, the presence of 2- and 3-MCPD fatty acid esters in food was mentioned in isolation without this finding suggesting a general presence in relevant food groups [Velisek et al.: 1980, Gardner et al.: 1983, Cerbulis et al. 1984]. Only in 2004 to 2006 was the finding gradually asserted that 3-MCPD fatty acid esters were present in the mg/kg range in refined vegetable oils and fats, and in proportional quantities in food that contain these products [Divinova et al. 2004, Svejkovska et al.: 2004, Zelinkova et al.: 2006]. In subsequent years, there was a rapid increase in knowledge. In addition to bound 3-MCPD, glycidyl fatty acid esters were also identified and found as further process contaminants in refined oils and fats, deodorisation during fat and oil refining representing the primary source for the formation of the bound analytes [Weißhaar: 2008a, Weißhaar et al.: 2010, Hrncirik et al.: 2011, Ermacora et al.: 2014]. The presence of relevant quantities of fatty acid-bound 2-MCPD and the associated occurrence of bound 2- and 3-MCPD with bound glycidol in a range of refined vegetable and animal oils and fats was reported in 2011 [Kuhlmann: 2011]. The formation mechanisms that lead to the development of the MCPD and glycidyl fatty acid esters have not yet been fully proven, but rather they represent working hypotheses [Hamlet et al.: 2010, Destaillats et al.: 2012a, 2012b]. Models that form the basis of a temperature-induced formation of MCPD esters from cyclic acyloxonium ions [Rahn et al.: 2011a, 2011b], for example, contradict the hypothesis of radical formation [Zhang et al.: 2013]. It appears

that during the refining of palm oil, 3-MCPD fatty acid esters are predominantly formed from di- and triacylglycerides, whereby a multitude of inorganic or thermolabile organic compounds can occur as a source of chloride. In comparison, glycidyl esters seem to form at somewhat higher temperatures as 3-MCPD esters from mono- and diacylglycerides as a result of elimination reactions [Destaillats et al.: 2012a, 2012b]. There has been less research to date into the formation of the fatty acid-bound 2-MCPD. It is plausible that the path of formation proceeds primarily via 3-MCPD fatty acid esters, which then isomerise at the standard deodorisation temperatures and lead to a more or less constant ratio of about two parts 3-MCPD to one part 2-MCPD, as found in most refined oils and fats [Hamlet et al.: 2010, Kuhlmann: 2016]. This theory is also supported by the fact that the formation of 2-MCPD at lower temperatures such as during the acidic hydrolysis of plant proteins [van Bergen et al.: 1992] or when roasting, baking or frying, as well as those used in the gentle deodorisation of heat-labile fish oils, occurs at a significantly lower rate compared with the formation of 3-MCPD.

3.3) Analytics

In recent decades, a complex range of analytical methods have been developed to determine MCPD, its derivatives and related compounds, including glycidyl esters. While the determination of free 3-MCPD in soy sauce and relishes and in a range of compound foods has been long established and even validated, at least for 3-MCPD [AOCS Official Method 2000.1, Method L 52.02-1 of the official collection of test methods pursuant to § 35 of the German Food Code, LMBG], according to our knowledge there is no validated method to determine free 2-MCPD in food. The same applies to the application of traditional methods to determine free MCPD, which are conventionally performed in a process that contains chloride and have not been tested for the possible formation of artefacts in the food matrices being studied. From an analytical perspective, it is highly complicated to quantitatively determine the bound analytes because originally it does not relate to three individual substances but in each case a group of fatty acid-bound homologous or isomeric compounds. In addition, 3-MCPD- and glycidyl derivatives may convert into each other depending on the pH. In an alkaline environment, 3-MCPD is unstable and can be quickly converted into glycidol whereas in an acidic environment, glycidol usually reacts with an epoxy ring opening. If strong nucleophiles such as halide ions are present at the same time, halogenated propanediols such as MCPD or the homologous monobromopropanediol (MBPD) are primarily formed. MCPD fatty acid esters can occur as mono- or diesters and it is likely that all the fatty acids

present in a refined oil or fat are also represented in the MCPD derivatives. Including all the stereo isomers, this means that in the presence of, for instance, six relevant fatty acids in an oil, 96 3-MCPD derivatives would need to be considered [Dingel: 2013]. Due to its symmetrical structure, 2-MCPD with the same number of fatty acids provides a lower number of possible derivatives, whereas glycidol can only occur in the form of fatty acid monoesters, making the number of glycidyl derivatives identical to the number of fatty acids. Fundamentally, it should be considered that, for diesters from two different fatty acids, there are three isomeric compounds with the same molar mass in each case. For instance, in addition to the 2-MCPD derivative, there are two 3-MCPD esters in which the fatty acids are alternately in positions 1 and 2. Even in the case of the diesters, the compounds are relatively large molecules that are not ideally suited to gas chromatography analysis as a result of their low volatility and instability in the case of the glycidyl esters. For this reason, the so-called **direct analysis** of the intact MCPD- and glycidyl fatty acid esters mainly developed using liquid chromatography mass spectrometry (LC-MS) techniques [Crews et al.: 2013]. Nevertheless, there are no methods that cover all three analyte groups at once whilst also being able to separate all the isomeric MCPD esters (MacMahon et al: 2013a, 2013b]. The principle restrictions of the liquid chromatography analysis of MCPD- and glycidyl esters particularly relate to the low chromatographic selectivity compared with gas chromatography (GC) in the multi-analyte method, which reaches its limits when multiple isomeric compounds with similar fragmentation patterns but varying signal intensities (response) need to be detected. Furthermore, the LC-MS methodology is exceptionally susceptible to matrix effects and frequently displays the effect of signal suppression related to substance quantity (quenching). Quantifications are generally performed using stable-isotope-labelled internal standards to compensate for this effect. In the case of the direct analysis of MCPD and glycidyl esters, only a limited selection of reference substances is currently commercially available and there are even fewer isotope-labelled standards. Consequently, according to the current assessment, oils and fats with short-chain fatty acids (e.g. coconut oil, milk fat), various unsaturated fatty acids (e.g. fish oils) or rarer fatty acids (speciality oils) cannot be analysed using direct LC-MS methods for MCPD or glycidol contents. A general disadvantage of the direct analysis of bound MCPD and glycidol is that the analytes are lipophilic and therefore very similar to the oil and fat matrix. This makes it difficult and correspondingly laborious to perform the necessary matrix separation in chromatographic trace analysis. For example, the most effective direct LC-MS method at present involves a

quadruple solid phase extraction and two separate analysis runs for each sample [MacMahon, 2013a, 2013b]. The advantages of direct analysis lie primarily in the extent of information when determining the analytes in their original form. This may permit conclusions on the formation pathways of the analytes and would be important if the toxicology of the individual esters or ester groups had to be assessed differently. The suggestion that artefact formations have to be excluded using this technology has not been fully confirmed. For instance, in certain ionisation techniques, conversions of 3-MCPD mono esters into glycidyl esters were observed in the analysis system [MacMahon, 2013a]. As a result of the many limitations, no direct method for the parallel determination of 2- and 3-MCPD fatty acid esters and glycidyl esters could be officially validated to date.

The so-called **indirect analysis** to determine 3-MCPD was used at a very early stage of discovery of the presence of 3-MCPD fatty acid esters. Oils or fats are transesterified using well-known and proven methods to release the analytes from their bound form. In contrast to the fatty acid esters, the free compounds are relatively polar as a result of their hydroxyl groups and therefore readily soluble in water. This makes it easy to achieve the necessary separation of lipophilic matrix components via liquid-liquid extraction. The relative volatility of the free chloropropanediols makes determination via gas chromatography simple. Usually, derivatisation methods are used to mask the hydroxyl functions and thereby improve the gas chromatographic properties of the analytes and achieve high measurement sensitivity. The use of phenylboronic acid (PBA) is now established out of a large number of derivatisation reagents used to date, because this reagent, unlike many others, is not sensitive to hydrolysis or matrix effects. Another particular advantage is that phenylboronic acid only reacts with diols such as 2-MCPD and 3-MCPD to form stable derivatives whereas monohydroxy compounds or those with other active groups such as amines, carboxylic acids, thiols etc. do not react in a stable manner. As these compounds are often present in food and often cause chromatographic interference, it is possible to further purify them via the derivatisation reaction with PBA, which makes indirect analysis relatively robust and resistant to matrix effects. The initial determinations of bound 3-MCPD in the lipophilic extracts of food and a little later in refined vegetable oils and fats were performed using an acid-catalysed transesterification, which required relatively long reaction times of 16 hours. Derivatisation with PBA took place in acidified aqueous sodium chloride solution in heat. Following this, the derivatised analytes were extracted from the solution and measured using GC-MS [Divinova et al.: 2004; Zelinkova et al.: 2006]. Soon after, a method was introduced to

determine 3-MCPD fatty acid esters in oils and fats, which was based on a base-catalysed transesterification whereby the reaction time could be limited to 10 minutes. Again, acidified sodium chloride solution was used to remove the matrix and in the derivatisation with PBA at 80 °C in acidic aqueous solution. The derivatised analyte was then extracted and measured using GC-MS [Weißhaar: 2008b]. This methodology was validated by the German Society for Fat Science (DGF) in 2009 as a DGF Standard Method [DGF C-III 18(09)]. It can be called the "*old Weißhaar method"*. However, at this time the occurrence of glycidyl esters associated with 3-MCPD was unknown. But these compounds were similarly cleaved which means that the released glycidol in the acidic environment could react in a virtually quantitative process with the chloride ions that were also present to form induced MCPD, thereby resulting in overestimations or false positive findings for 3-MCPD [Weißhaar: 2010]. This issue was irrelevant to the acid-catalysed transesterification because the glycidol function is already destroyed during treatment with the acid. As a result, the DGF Method C-III 18 (10) was modified such that two sample aliquots were analysed in parallel. One aliquot was treated with methanolic sulphuric acid before ester hydrolysis to destroy the glycidyl function before further analysis. The result therefore should represent just the 3-MCPD esters while the second sample aliquot was prepared without acid treatment and therefore represented the total of bound glycidol and 3-MCPD. In this case, the 3-MCPD result was indistinguishably composed from the original compound contained in the sample and the 3-MCPD induced by glycidol release and transformation. By means of a double determination, which can be called the "*Weißhaar differential method*", it was possible to mathematically determine the glycidol. The conversion of the glycidol into 3-MCPD was deemed quantitative and accounted for using a stoichiometric factor of 0.67 that correlated the different molecular weights of the two compounds. The quantification was initially achieved by using isotope-labelled free d5-3-MCPD as an internal standard and external calibration after measuring via GC-MS. A d5-3-MCPD di-fatty acid ester was subsequently introduced as an internal standard. Unfortunately, the considerable changes to the method initially led to neither a newly identified method nor any clear specification to identify from which method variation the reported 3-MCPD results came. This also meant that from the present perspective, results obtained using the former methodology are difficult to assess and potentially should not be used for exposure assessments. In attempting to validate the "*Weißhaar differential method*" it was shown that the acidic pretreatment for the purpose of destroying the glycidyl esters either did not proceed completely or caused artefacts to form, which meant that false high findings of 3-MCPD were

recorded in this part of the method. Consequently, there was no longer any reliable guarantee of calculated glycidol content, which could lead to false-low findings for this analyte. As a result, this part of the method was withdrawn by the Joint Committee for the Analysis of Fats, Oils, Fatty products, Related Products and Raw Materials (GA Fett) and only the total determination was validated as Standard Method C-VI 17 (10). This method is called the "*Weißhaar method*" [GA Fett: 2012].

At the same time, another indirect, GC-MS-based differential method was developed with base-catalysed ester hydrolysis and PBA-derivatisation to analyse 3-MCPD fatty acid esters in oils and fats. Part A of the method is used to determine the total 3-MCPD and glycidol (expressed as 3-MCPD) by treating the transesterification products with sulphuric sodium chloride solution. Part B of the method is based on a chloride-free work-up, in which the glycidol that is released via the possible presence of glycidyl esters, cannot be converted into induced 3-MCPD but rather reacts in other ways. This results in just the original 3-MCPD content being determined and the glycidol can be calculated from the difference of the two results if the conversion of glycidol to 3-MCPD in part A of the method is empirically determined. This empirical determination of the glycidol transformation should take into account that, in the aqueous acidic environment, glycidol can react to form 2-MCPD or glycerin as well as 3-MCPD or the conversion may not be complete depending on the pH. In addition to the chloride-free work-up in part B and the determination of the real glycidol transformation, this analysis also differs from the "*Weißhaar method*" by the shorter time frame for transesterification for the purpose of avoiding substantial losses of 3-MCPD in the chloride-free preparation B. To minimise the risks of a possible artefact build-up and to avoid large reagent excesses, the derivatisation reaction with PBA is not performed in acidic sodium chloride solution with heat but instead in organic solvents at room temperature. Ultimately, this avoids measuring external calibration series and instead quantifying via an internal single-point calibration. The measurements are taken as part of Selected Ion Monitoring (SIM) whereby the analytes are detected on the daughter ion traces with a mass/charge ratio m/z - 147 (3-MCPD derivative) or m/z = 150 (d_5-3-MCPD derivative). Further daughter fragments and the molecular ions provide validation. This method was validated by the DGF in 2010 and is described as Standard Method C-VI 18 (10). The Joint Committee GA Fett also identifies this method as the "*Kuhlmann method*" [GA Fett: 2012]. In 2013, this method was validated for oils and fats by the American Oil Chemists Society (AOCS) together and in comparison with two other methods, and published as the AOCS Official Method Cd 29c-13

[AOCS: 2013c]. The advantages of Cd 29c-13 are that it is fast, due to the short transesterification process, and therefore results for process monitoring, for example, can be produced promptly, i.e. within a few hours. The DGF method validation has shown that free 3-MCPD contributes to the results, however, the values do not disclose whether or in what proportion free analyte was present in the samples. It can be considered a disadvantage that this method has not been validated for the determination of bound 2-MCPD because at the time of its development and initial validation, the relevant isotope-labelled internal standards were not available. Furthermore, the determination of the glycidyl ester content over two measurements causes increased measurement uncertainty. As the 3-MCPD measurement with its error tolerance is involved in both determinations, the precision of the glycidol determination reduces the lower the glycidol content in a respective sample is proportional to the 3-MCPD content. Finally, there is a loss of analyte in the chloride-free preparation in part B of the method causing the 3-MCPD to be converted irreversibly into glycidol. This makes this determination less sensitive than part A, in which the presence of chloride ensures a reverse reaction of glycidol into 3-MCPD.

The second method validated by AOCS to determine bound 2- and 3-MCPD and bound glycidol, the AOCS Official Method Cd 29b-132, is also known by the name "*SGS 3-in-1*" method. It represents a further development of the "*Kuhlmann method"*, in which the alkaline transesterification is performed under such mild conditions that the undesired transformation of 3-MCPD to glycidol is inhibited to a minimum level. To handle the disadvantages of the differential in the glycidol determination, the released glycidol is subsequently largely transformed in acidic solution with bromide into 3-MBPD. Compared with glycidol, 3-MBPD is stable and, as a result of its chemical homology, can be derivatised together with 2- and 3-MCPD in a one-pot synthesis using phenylboronic acid. In its original form, the "*SGS 3-in-1*" method is performed such that samples are each prepared in duplicate using various internal standards. In an assay A, d_5-2-MCPD, $d_5$3-MCPD and a d_5-glycidylester are used, in an assay B only a d_5-2-MCPD-di-fatty acid ester and a d_5-3-MCPD-di-fatty acid ester are used. The measurement from A thereby provides a raw value for glycidyl ester and results for 2- and 3-MCPD with a theoretical 100 % transesterification. Measurement B gives the results interpreted as true for 2- and 3-MCPD, the difference in the MCPD results between A and B can be used for the quantitative determination of the transesterification rate. Measurement B also enables determination of the signal for d_5-3-MBPD, which is produced by the d_5-3-MCPD-di-fatty acid ester. As its quantity is always known, this approach makes it possible to

individually determine the quantity of unwanted induced glycidol for each sample and therefore the correction of the glycidol raw value from measurement A. In terms of the procedure and measurements, the AOCS 29b-13 ("*SGS 3-in-1"*) method is performed in virtually the same way as the AOCS Cd 29c-13 ("*DGF-Kuhlmann*") method. Both the 2-MCPD derivatives and the 3-MBPD and d_5-3-MBPD derivatives representing the glycidol are detected in SIM mode in the molecular ion traces. The limit of quantification for all analytes is 100 µg/kg. [Kuhlmann: 2011; AOCS: 2013-b]. The disadvantages of the "*SGS 3-in-1"* method lie in a long transesterification period (16 h) and the effort incurred by the duplicate sample preparation and evaluation. Furthermore, although to a lesser extent than for the previous DGF method, there is also the limitation that the relative result uncertainty increases in the glycidol determination if there are very large 3-MCPD concentrations and very low quantities of glycidol. Free 2- and 3-MCPD contributes to the results because there is no separation of these compounds before ester hydrolysis. However, the results do not disclose whether or how much of the MCPD was present as a free compound in the samples. It is not possible to separate the free analytes by extraction after adding the internal standards because, as d_5-2-MCPD and d_5-3-MCPD in assay A, they are also used as free compounds and after extraction they would no longer be available to detect the rate of hydrolysis. The advantages of the methodology can be seen in the fact that along with the validated detection of the bound 2-MCPD, the glycidol is not detected using an error-prone differential method but by directly measuring the analytes. The detection sensitivity is also high for 3-MCPD because the loss of the analytes in the base-catalysed transesterification is negligible. The practical application of this method has shown that during routine analysis, the method is robust enough to omit duplicating every sample if only one sample per analytical sequence is investigated as an example of the induced glycidol formation and the resulting correction factor is used for the other measurements of the sequence. Duplicating the sample weight and adjusting the sample preparation also permits significantly lower limits of quantification of 10 µg/kg in each case for all analytes [Kuhlmann: 2016]. This method, which in terms of its robustness and the very low limits of quantification can be considered to be the most effective method at present, was used as part of the project "Investigation into the presence of 3-MCPD esters and related compounds in foods" to investigate all the oils, fats and margarines.

The third indirect method validated by the AOCS to determine MCPD and glycidyl esters in oils and fats, the AOCS Official Method Cd 29a-13 ("*Unilever"* method) was developed to circumvent the disadvantages of the base-catalysed transesterification and to establish an acid-

catalysed version as was used at the start of the analysis development for bound 3-MCPD. However, as glycidyl esters are unstable under these reaction conditions, they need to be stabilised in an upstream reaction step. In the "*Unilever*" method, this is achieved by an acid-catalysed transformation with bromide into the corresponding 3-MBPD fatty acid monoesters. Following extraction to isolate the lipid phase that contains the MCPD and MBPD fatty acid esters, this is then transesterified for 16 hours in the presence of an acid catalyst. The reaction is terminated by using an alkaline stopping reagent, the lipid matrix is removed by means of liquid-liquid extraction, and derivatisation is conducted using phenylboronic acid in acidic aqueous solution with ultrasonic treatment and subsequent re-extraction of the analytes. The quantification step proceeds by using isotope-labelled esterified analytes in the samples and an external calibration [Ermacora, 2013; AOCS Cd 29a-13]. Only preparing one sample in the AOCS Cd 29a-13 ("*Unilever*" method) makes it less laborious in terms of sample preparation and data evaluation than the two alternative AOCS methods despite the comparatively more complex calibration measurements. The time required for transesterification is relatively long and comparable with the "SGS 3-in-1" method. Theoretically, the result uncertainty for glycidol should be removed for high 3-MCPD contents because no transformation is expected during the acid-catalysed transesterification. The disadvantages of the AOCS Cd 29a-13 ("*Unilever*" method) include the particular risk of artefact formation, which could lead to false high findings. This has two potential causes. Firstly, the complete sample set with all known and unknown primary and subsidiary compounds and of course, all the trace elements, come into contact with sulphuric acid and bromide ions in an organic medium (tetrahydrofuran). In this combination, the bromide ions are expected to have especially strong nucleophilic power and active compounds in addition to the glycidyl esters could react to form derivatives or precursors of MBPD. A similar effect in the "*Weißhaar differential method*" led to false high findings of 3-MCPD and caused the DGF to classify the analysis as invalid. This risk is lower for the methods that use alkaline transesterification because the preceding transesterification should have destroyed a large number of reactive compounds. The next step in the alkaline method is the liquid-liquid extraction in which practically the entire lipid matrix and all the components that are not readily water soluble are removed, leaving just the water-soluble analytes. Ultimately the glycidol conversion to MBPD in the AOCS Cd 29b-13 ("*SGS 3-in-1*") method is consistently performed in an aqueous medium, whereby the bromide ions should be stabilised through strong solvation and should be less nucleophilic. The second reason why the AOCS Cd 29a-13 ("*Unilever*" method) is considered

more susceptible to artefact formation is that the acid-catalysed transformation of the glycidyl esters with 90 µg sodium bromide per batch only uses very small quantities of bromide. However, this also means that the naturally occurring chloride in the samples competes with the bromide and could cause the glycidyl esters or other reactive components to convert into 3-MCPD derivatives. This would result in corresponding false high findings of 3-MCPD. For these reasons, it can be assumed that the AOCS Cd 29a-13 ("*Unilever*" method) has higher limits of quantification than the methods validated at the same time and it is less robust in terms of the matrix composition compared with the methods based on alkaline ester hydrolysis. In fact, the statistical data of the AOCS method validation indicate that at very low concentrations of glycidyl esters, higher results can be achieved with the "*Unilever method*" than with the "*DGF method*" or the "*3-in-1 method*". As expected, the latter method provided the most precise data at the lowest recorded glycidol concentrations [AOCS: 2013a-c]. Furthermore, when developing or validating the methods there was no investigation into the extent of which free MCPD contributed to the results. The free analytes may only play a marginal role in freshly refined oils and fats, however, they may be present in relevant quantities in used frying fats and in fat extracts of compound foods.

While the analysis of bound MCPD and glycidol in vegetable oils and fats was considered to be established and validated at the time of the project, this did not apply to the same analysis in compound foods. In 2013, the BfR validated a method for the determination of 2- and 3-MCPD esters in compound foods such as chocolate and strawberry pudding, milk powder, mayonnaise and vegetarian onion spread. This method was based on extracting fat, preferably via Accelerated Solvent Extraction (ASE) with *tert*-butyl methyl ether (tBME) and subsequent alkaline transesterification at room temperature as well as chloride-free extraction and analyte derivatisation in a similarly way to part B of the DGF method C-VI 18 (10). Although this method did not take into account the determination of the glycidyl esters and did not include relevant matrices such as pasta products, differentiation was made between free and bound analytes in the extraction. This showed that ASE with tBME almost entirely extracted the bound analytes while the recovery of free 3-MCPD was less than 40 % [Fry et al.: 2013].

In 2015, the Joint Research Centre of the EU (JRC) introduced a method that was validated in-house for the separate determination of free 2- and 3-MCPD, bound 2- and 3-MCPD, and bound glycidol in a variety of foods including pasta products, fish and meat products, and French fries. The methodology covered, in a separate work-up, the analysis of the free MCPD

isomers after extraction, derivatisation using PBA and GC-MS measurement. For the bound analytes, a method presented as "*pressurised liquid extraction*" (PLE) was chosen in which the samples were extracted using tBME in a pressure-supported ASE machine. However, the temperature of 40 °C was kept considerably below the boiling point of the solvent so the ASE conditions were not conventional. This method was proved to be unsuitable for infant formula in the method validation with recovery of approx. 20 %. Aside from this, the further analysis was based essentially on the application of the AOCS Cd 29a-13 ("*Unilever*" method) on the isolated fat. Modifications to the method included adding the isotope-labelled internal standard at a sample fat proportion > 5 % after extraction. The derivatisation using PBA was also not performed in an aqueous solution but in organic solvents similarly to the "SGS 3-in-1" analysis [Wenzl et al.; 2015]. The advantages of this method are that a methodology was developed that officially conformed to the requirements of the EFSA recommendation for monitoring all specified free and bound analytes. The limits of quantification at a range of 13 µg/kg (3-MCPD ester) to 31 µg/kg (glycidyl ester), each based on the fat phase, were also exceptionally low. This does need to be qualified by pointing out that the limits of quantification were calculated from doping experiments with pure blank oils and not by determining the signal-to-noise ratios in complex compound foods. Certain restrictions to the method can be seen in that the methodology is relatively cumbersome at certain points for a routine analysis. The limitations of aqueous solutions for drying when determining the free MCPD are also a restriction along with the addition of the internal standard before or after extraction depending on the fat content of the samples. Performing the extraction of samples with a fat content > 5 % could be considered critical because this does not compensate for extraction losses. It is considered critical that the method validation does not show whether it guarantees complete separation of the free analytes from the bound analytes. While carrying over the bound analytes to the analysis of the free compounds does not seem problematic because the intact esters do not interfere with the determination of the free compounds as a result of their fundamentally different properties in terms of chromatography-mass spectrometry, carrying over the free analytes to the bound analyte fraction would contribute to false high readings. However, the fact that free MCPD is proportionately extracted with tBME was proven in BfR studies [Fry et al.: 2013]. It is also considered critical that the methodology cannot be used for infant formula (milk powder) and that the AOCS Cd 29a-13 ("*Unilever*" method), which is suspected to be susceptible to matrix effects, is part of the procedure. Even when extracting compound foods, it is assumed that a large number of other

substances are isolated along with the lipids and therefore the number of substances that interfere with the glycidyl ester transformation may increase significantly.

While the project work was being carried out, the AOCS performed a method validation for the determination of bound MCPD and glycidol in oil-based emulsions such as margarine or mayonnaise. Nevertheless, there was still no official method at the end of this project. As this method was only developed for a highly specific product group and possible interferences from free MCPD was not part of the scope of validation, it was not considered any further for the project presented in this paper.

To circumvent as far as possible all the outlined limitations of the aforementioned methods for the determination of free 2- and 3- MCPD along with the MCPD esters and glycidyl esters in compound foods, a method was developed and validated in-house as part of this project with which all the relevant compound food groups can be analysed at the same time. It consists of a three-stage Heat-Ultrasonic-Pressure-supported Solvent Extraction (HUPsSE) in which polar and non-polar solvents are used and the obtained extracts are combined. After separating all the used solvents, conventional liquid-liquid extract is used to separate the polar from the non-polar fractions of the extract. In so doing, the non-polar fraction is enriched in a mixture of isohexane/tBME. The bound analytes are readily soluble whereas under the work-up conditions the free analytes are virtually never carried over into the non-polar phase. The polar fraction is analysed without chloride for free 2 and 3-MCPD. A simplification of the "3-in-1" analysis is used to avoid ester hydrolysis or glycidol determination. By successfully utilising FAPAS laboratory comparative studies, this method has been tested for many years for its suitability for the analysis of soy sauce and protein hydrolysates, the only laboratory comparison matrix available at this time. For the determination of the 3-MCPD esters and related compounds, the non-polar fraction is analysed using a modified "3-in-1" analysis for fatty acid-bound 2 and 3-MCPD and glycidyl esters. The modifications, as for the methods for oils and fats used as part of this project, relate to adapting the fat quantity used as well as omitting the duplicate preparation for the representative determination of the induced glycidol formation. This makes it possible to firstly dope the samples with all isotope-labelled free and bound internal standard compounds before the HUPsSE and therefore compensate for any losses or matrix influences across all sample preparation steps. Secondly, the bound analytes can also be determined after separating the free analytes using the specified modifications and the methodology of the AOCS 29b-13 (SGS "3-in-1") method, which is regarded as being especially robust and sensitive. In so doing, limits of quantification based on the total sample

set of 5 µg/kg could be achieved for the free analytes and 10 µg/kg in each case for the bound analytes. In comparison to all the other currently available parallel methods, this concept enables the greatest compliance with requirements for current analysis, namely a method that is as uniform as possible, highly comparable, that can be applied for as many compound foods as possible, and that separately and sensitively records the free and bound analytes. Furthermore, the "*3-in-1*" analysis is designed in such a way for oils and fats, as well as for compound foods, that, in comparison with the JRC method or AOCS Cd 29a013 "*Unilever*" method, significantly lower quantities of solvents and chemicals are used – a concept that is also positive and up-to-date in terms of sustainability.

4) Materials and methods

4.1) Reagents and chemicals

3-MCPD: 99 %, Dr. Ehrenstorfer GmbH (Augsburg, Germany).

d_5-3-MCPD: 98 %, Sigma-Aldrich (Steinheim, Germany).

2-MCPD: 98 %, Toronto Research Chemicals Inc. (Toronto, ON, Canada).

d_5-2-MCPD: ≥ 98 %, Toronto Research Chemicals Inc. (Toronto, ON, Canada).

3-MCPD-1,2-dioleoyl ester: 98 %, Toronto Research Chemicals Inc. (Toronto, ON, Canada).

d_5-3-MCPD-1,2-dioleoyl ester: 98 %, Toronto Research Chemicals Inc. (Toronto, ON, Canada).

2-MCPD-1,3-dipalmitoyl ester: 98 %, Toronto Research Chemicals Inc. (Toronto, ON, Canada).

d_5-2-MCPD-1,3-dipalmitoyl ester: 98 %, Toronto Research Chemicals Inc. (Toronto, ON, Canada).

Glycidyl oleate 98 %, Toronto Research Chemicals Inc. (Toronto, ON, Canada).

d_5-glycidyl oleate: 98 %, Toronto Research Chemicals Inc. (Toronto, ON, Canada).

Methanol: p.a. or better, Merck (Darmstadt, Germany).

tert-Butyl methyl ether: p.a. or better, Merck (Darmstadt, Germany).

Diethyl ether: p.a., Merck (Darmstadt, Germany).

Toluene: p.a., Merck (Darmstadt, Germany).

Isohexane (2-methylpentane): p.a., Merck (Darmstadt, Germany).

Isooctane (2,2,4-trimethylpentane): for pesticide residue analysis, Fluka - Sigma-Aldrich (Steinheim, Germany).

Ethyl acetate (acetic ester, ethyl ethanoate, EE): p.a., Merck (Darmstadt, Germany).

Water: Millipore grade, < 20 µS/cm, < 5 ppb TOC

Phenylboronic acid:≥97 %, Sigma-Aldrich (Steinheim, Germany).

Sodium sulphate: granulated, for organic trace analysis, Merck (Darmstadt, Germany)

Before use, the sodium sulphate was baked out over night in a muffle furnace at approx. 500 °C.

Sodium bromide: Ph Eur, Merck (Darmstadt, Germany).

Sodium hydroxide: 97 %, 20-40 mesh, Sigma-Aldrich (Steinheim, Germany).

ortho-phosphoric acid: 85 %, Merck (Darmstadt, Germany).

Nitrogen: 4.0 or better, Linge AG (Pullach, Germany)

Helium: 4.0 or better, Linge AG (Pullach, Germany)

4.2) Chemical solutions and reagents

Solution 1: 6 g sodium hydroxide in 100 mL methanol.

Solution 2: 2.5 g sodium hydroxide in 100 mL methanol.

Solution 3: aqueous sodium bromide solution (600 g/l) acidified with 6.5 mL/l *ortho*-phosphoric acid. 1.2 mL of this solution should just acidify 700 µL of solution 1 beyond the neutral point. If this was unsuccessful after freshly preparing the solutions, the acid solution was adjusted by diluting or by adding more *ortho*-phosphoric acid to solution 1.

Solution 4: aqueous sodium bromide solution (600 g/l) acidified with 2.7 mL/l *ortho*-phosphoric acid. 1.2 mL of this solution should just acidify 700 µL of solution 2 beyond the neutral point. If this was unsuccessful after freshly preparing the solutions, the acid solution was adjusted by diluting or by adding more *ortho*-phosphoric acid to solution 2.

Solution 5: saturated aqueous sodium sulphate solution (supernatant above sediment).

Solution 6: aqueous sodium bromide solution (approx. 600 g/l).

Solution 7: phenylboronic acid in diethyl ether: approx. 5 mg/ml

4.3) Stock and working solutions

In general, reference substances and isotope-labelled standards were weighed in their solid form and diluted to prepare stock solutions with methanol (free analytes) or toluene (bound analytes) to a concentration of 1 mg/ml or 100 µg/ml. These concentrations are always based on the core analytes 2-MCPD, 3-MCPD and glycidol. This means that increases in the molecular weight as a result of isotope labelling or esterification were taken into account by proportionally higher weights. Mixed working solutions were prepared from these stock solutions under gravimetric control, these working solutions containing either the free analytes dissolved in methanol or the bound analytes dissolved in toluene.

Working solution 1: 2-MCPD and 3-MCPD, 10 µg/mLof each in methanol.

Working solution 1d: d_5-2-MCPD and d_5-3-MCPD, 10 µg/mLof each MCPD equivalent in methanol.

Working solution 2: 2-MCPD and 3-MCPD, 1.0 µg/mLof each in methanol.

Working solution 2d: d_5-2-MCPD and d_5-3-MCPD, 1.0 µg/mLof each MCPD equivalent in methanol.

Working solution 3: 2-MCPD-1,3-dipalmitoyl ester, 3-MCPD-1,2-dioleoyl ester and glycidyl-oleate with 5 µg/mL2-MCPD equivalent, 10 µg/mL3-MCPD equivalent and 5 µg/mLglycidol equivalent in toluene.

Working solution 3d: d_5-2-MCPD-1,3-dipalmitoyl ester, d_5-3-MCPD-1,2-dioleoyl ester and d_5-glycidyl oleate with 5 µg/mL d_5-2-MCPD equivalent, 10 µg/mL d_5-3-MCPD equivalent and 5 µg/mL d_5-glycidol equivalent in toluene.

Working solution 4: 2-MCPD-1,3-dipalmitoyl ester, 3-MCPD-1,2-dioleoyl ester and glycidyl-oleate with 0.5 µg/mL 2-MCPD equivalent, 1.0 µg/mL 3-MCPD equivalent and 0.5 µg/mL glycidol equivalent in toluene.

Working solution 4d: d_5-2-MCPD-1,3-dipalmitoyl ester, d_5-3-MCPD-1,2-dioleoyl ester and d_5-glycidyl oleate with 0.5 µg/mL d_5-2-MCPD equivalent, 1.0 µg/mL d_5-3-MCPD equivalent and 0.5 µg/mL d_5-glycidol equivalent in toluene.

Working solution 5d: d_5-3-MCPD-1,2-dioleoyl ester, 10 µg/mL d_5-3-MCPD equivalent in toluene.

Working solution 6: glycidyl oleate, 1 µg/mLin toluene.

4.4) Equipment and consumables

2 mL crimp cap vials & crimp caps with Teflon seal: Wicom Germany GmbH (Heppenheim, Germany)

Inserts for 2 mL crimp cap vials, approx. 200 µL internal volume:

VWR International GmbH (Darmstadt, Germany).

These inserts were baked out overnight in a muffle furnace at approx. 500 °C.

4 mL screw cap vials & screw caps with Teflon seal: Wicom Germany GmbH (Heppenheim, Germany)

8 mL screw cap vials & screw caps with Teflon seal: Wicom Germany GmbH (Heppenheim, Germany)

12 mL screw cap vials & screw caps with Teflon seal: Wicom Germany GmbH

(Heppenheim, Germany)

Pasteur pipettes: VWR International GmbH (Darmstadt, Germany).

Bulbs for Pasteur pipettes, 2 mL internal volume: VWR International GmbH (Darmstadt, Germany).

Eppendorf pipettes, various sizes: Eppendorf AG (Hamburg, Germany)

Blow-off devices, pressure-regulated nitrogen dispenser with replaceable outlets (Pasteur pipettes) and temperature-regulated heating plate: SGS Germany GmbH own design (Hamburg, Germany)

Ultrasonic bath, programmable temperature and time: USC-THD/HF, VWR International GmbH (Darmstadt, Germany).

Vortex mixer: e.g. lab dancer S40, VWR International GmbH (Darmstadt, Germany).

Centrifuge, max. 4000 rpm: Rotofix 32 A: Andreas Hettich GmbH & Co KG (Tuttlingen, Germany)

Knife mill: Grindomix GM 200, Retsch GmbH (Haan, Germany)

Disperser: Ultra Turrax T25 with S25N-8g disperser tool (8 mm outer diameter of stator), Janke & Kunkel GmbH & Co KG (Staufen, Germany)

Gas chromatograph: 7890A: Agilent Technologies (Santa Clara, California, USA)

Mass spectrometer: 5975C inert XL: Agilent Technologies (Santa Clara, California, USA)

4.5) Sampling

The food samples were purchased within the period February to September 2016. As far as possible and meaningful, the products were purchased in proportion to the market shares of the food retailers. The code for the market shares was taken from the latest available statistical survey [LMZ: 2015]. Infant formula and follow-on formula were excluded from this method because the intention here was to obtain up to 10 batch samples of all brands available on the German market. Table 1 lists the specific brand batch numbers.

Infant formula brand 1	**Batch number**
A-1	10
B-1	10
C-1	10
D-1, organic	10
E-1, organic	10
G-1, organic	10
H-1	10
I-1	10
J-1, organic	10
K-1	6
L-1	10
M-1	10
N-1	3
PRE infant formula brand	**Batch number**
A-PRE	10
B-PRE	10
C- PRE	10
D-PRE, organic	10
E-PRE, organic	10
G-PRE, organic	10
I-PRE	10
K-PRE	10
L-PRE	10
M-PRE, organic	10
N-PRE	2
Total number of samples	**221**

Table 1: Overview of sampling for infant formula.

The following tables 2 and 3 show the commodity codes for the further product groups:

Commodity codes, edible oils			
Oil type	**Sample number**	**Type**	**Sample number**
Rapeseed oil	50	standard	40
		organic	10
Sunflower oil	58	standard	40
		organic	18
Corn oil	10	standard	10
Olive oil	20	standard	10
		organic	10
Sesame oil	10		
Peanut oil	10		
Safflower oil	3		
Hazelnut oil	3		
Walnut oil	3		
Linseed oil	3		
Grapeseed oil	4		
Pumpkin seed oil	1		
Soybean oil	2		
Red palm oil	2		
Wheat germ oil	1		
Total number of samples	**180**		
Commodity codes, margarine			
Margarine	**Sample number**	**Type**	**Sample number**
Solid	180	Full fat and 66 %	112
		1/3 fat or 41–65 %	5
		1/2 fat	54
		Low fat	9
Liquid (vegetable fat)	20		
Total number of samples	**200**		
Commodity codes, frying fats			
Frying fat	**Sample number**		
Coconut oil	5		
Other	45		
Total number of samples	**50**		

Table 2: Overview of sampling, edible oils, margarine, frying fats.

Commodity codes, French fries			
French fries	**Sample number**		
Frozen products	34		
Takeaway purchase	66		
Total number of samples	**100**		
Commodity codes, croissants & doughnuts			
Croissants & doughnuts	**Sample number**	**Type**	**Sample number**
Croissants	100	Retail	59
		Takeaway	41
Doughnuts	50	Retail	25
		Takeaway	25
Total number of samples	**150**		
Commodity codes, spreads			
Spreads	**Sample number**	**Type**	**Sample number**
Sweet spreads	50	Nut nougat cream	43
		Other	7
Savoury spreads	50	Vegetarian without lard	15
		Lard spread (dripping)	4
		Cream cheese spread	23
		Other	8
Total number of samples	**100**		
Commodity codes, Asian dishes with instant noodles			
Asian dishes	**Sample number**		
With instant noodles	50		
Total number of samples	**50**		

Table 3: Overview of sampling compound foods.

4.6) Sample preparation of oils, fats, margarine

Sample preparation was modularly designed for various matrices so that multiple work steps were identical even for different analyses. For sections 4.6 and 4.7, “extracted”, “shaken” or “washed” therefore means that a liquid mixture was blended for a few seconds manually by vigorously hitting against the heel of the hand or by using a vortex mixer. When centrifuging, the process was accelerated for 1 minute in each case to 3000 to 4000 revolutions per minutes (rpm). New screw cap glassware and Pasteur pipettes were always used. When using Pasteur pipettes, an exception could be made to this if identical work steps were performed consecutively.

4.6.1) Sample preparation of oils, fats, margarine to determine free 2- & 3-MCPD

1.99 g to 2.01 g of the sample was each weighed in a 12 mL screw cap vial. Solid oils and fats were melted for this purpose, margarine in contrast was weighed as a solid to prevent separation. 100 µL of the standard working solution 2d (d_5-2-MCPD and d_5-3-MCPD, in each case 1.0 µg/mLMCPD equivalent in methanol) and 4 mL of a mixture of isohexane/tBME 4:1 (v,v) was added to each sample. The mixture was shaken in a sealed vessel until the lipid phase was completely dissolved and subsequently extracted twice using 2.5 mL of an aqueous 60 % sodium bromide solution (solution 6) in each case. After centrifuging, the aqueous phases were separated using a Pasteur pipette, combined in an 8 mL screw cap vial and washed at least once or twice with 2.5 mL of an isohexane/tBME mixture 4:1 (v,v), whereby the organic phase was to be separated using a Pasteur pipette and discarded. The aqueous phase was then extracted three times using 2 mL diethyl ether or a mixture of diethyl ether/ethyl acetate in each case 9:1 (v,v). The organic phases were each separated following centrifugation using a Pasteur pipette, combined in an 8 mL screw cap vial with a spatula tip of sodium sulphate, 100 µL of phenylboronic acid solution (solution 7) added and concentrated to dryness under a stream of nitrogen. For the subsequent GC-MS measurement, the soluble residue was resorbed in 300 µL isooctane and a 200 µL aliquot was transferred to a GC insert. Each analytical sequence included both a blank sample and a reference sample with a known analyte content.

4.6.2) Sample preparation of oils, fats, margarine to determine bound and free 2- & 30 MCPD and bound glycidol

The sample preparation to determine bound and free 2- and 3-MCPD and bound glycidol in oils and fats has been described already elsewhere [Kuhlmann, 2016]. In summary, the preparation consists of the weighted sample 200 mg +/- 1 mg in a 4 mL screw cap vial, the addition of 200 µL of the standard working solution 4d (*with 0.5 µg/mLd_5-2-MCPD equivalent, 1.0 µg/mLd_5-3-MCPD equivalent and 0.5 µg/mLd_5-glycidol equivalent in toluene*) and dissolving the preparation in 1.5 mL tBME. The base-catalysed transesterification was initiated by adding 700 µL of a methanolic sodium hydroxide solution (solution 1) at -26 °C and terminated after 15 h to 18 h by adding 1.2 mL of an acidic stopping reagent (solution 2). If the transesterification was conducted over the weekend, diluted reagent solutions (solution 2 and 4) were used accordingly; the reaction time then equated to 62 h to 66 h. To isolate the analytes, after removing the volatile solvent in the nitrogen stream, the reaction preparations were washed multiple times with 1 mL isohexane in each case or the same quantity of an isohexane/tBME mixture 4:1 (v,v). Pasteur pipettes were used to separate and discard the organic phases. The largely matrix-free aqueous phase was then extracted three times using 2 mL diethyl ether or a diethyl ether/ethyl acetate mixture in each case 9:1 (v,v). The resulting organic phases were each separated following centrifugation using a Pasteur pipette, combined in an 8 mL screw cap vial with a spatula tip of sodium sulphate, 30 µL of phenylboronic acid solution (solution 7) added and then concentrated to dryness under a stream of nitrogen. For the subsequent GC-MS measurement, the soluble residue was resorbed in 300 µL isooctane and a 200 µL aliquot was transferred to a GC insert. Each analytical sequence included both a blank sample (extra virgin olive oil) and a reference sample with a known analyte content. To determine the undesired transformation of 3-MCPD into glycidol, a so-called transformation sample was worked up for each analytical sequence. At the start of the work-up of the transformation sample, the standard working solution 4d was not used, instead 100 µL of each of the standard working solutions 5d and 6 (*d_5-3-MCPD-1,2-dioleoyl ester, 10 µg/mLd_5-3-MCPD equivalent in toluene and glycidyl oleate, 1 µg/mLglycidol equivalent in toluene*) was added.

4.6.3) Sample preparation using the "5-in-2" method for compound foods for the parallel determination of free 2- & 3-MCPD as well as bound 2- & 3-MCPD and bound glycidol

1.99 g to 2.01 g of a compound sample (infant formula, croissants, doughnuts, French fries, Asian ready meals with dried noodles, spreads) was weighed in a 12 mL screw cap vial. Homogeneous foods such as infant formula and spreads were weighed directly, heterogeneous foods such as croissants, doughnuts and French fries were ground up homogeneously using a knife mill prior to weighing. For Asian ready meals with dried noodles, additional package inserts such as spice and oil mixes were included in the homogenisation. Added to each sample was 100 µL standard working solution 2d (*with 1.0 µg/mLMCPD equivalent in methanol*) and 200 µL standard working solution 3d (*with 5 µg/mLd_5-2-MCPD equivalent, 10 µg/mLd_5-3-MCPD equivalent and 5 µg/mLd_5-glycidol equivalent in toluene*) and subsequently 6 mL +/- 1 mL methanol. Each sample of infant formula was thoroughly mixed to a paste, individually and immediately after methanol addition, to minimise clumping later. All containers in an analytical sequence were then tightly sealed and extracted by placing them horizontally in a plastic rack for 15 min in the ultrasonic bath at a starting temperature of 65 °C. After centrifuging, the liquid supernatant of each sample was transferred using a Pasteur pipette to a new 12 mL screw top vial and placed under a nitrogen stream on a base plate tempered to 70 °C in order to remove the solvent. While the organic phase of a sample was under the nitrogen stream, the corresponding solid residue from the first extraction step was added to 6 mL of a methanol/tBME 1:1 (v,v) mixture and mixed to a paste. If a powdered milk sediment clumped after the first HUPsSE treatment, an attempt was made to manually break up the sediment, for example using a spatula, after adding the next extraction solvent. If it was still impossible to achieve a fine-grained slurry, this was achieved by using a disperser at 24000 rpm with a particularly small stator to fit the reaction vessel. Similarly to the first extraction, the second was performed for 15 min in the ultrasonic bath at a starting temperature of 65 °C to 70 °C. The supernatant was then centrifuged and separated in the same way as in the first extraction step, and combined with the residue of the first supernatant, which was still under the nitrogen stream. Finally, a similar extraction was performed with 6 mL tBME, whereby the starting temperature of the ultrasonic bath could be between 65 °C and 73 °C. The resulting supernatant was added to the residues of the previously combined and corresponding extracts still under the nitrogen stream and the solution was concentrated approximately to dryness. The polar fraction was separated from the non-polar fraction by adding 4 mL saturated sodium sulphate solution (solution 5) and a

second extraction of this mixture with 2.5 mL of an isohexane/tBME 4:1 (v,v) mixture. The further preparation of the clean organic isohexane/tBME fraction, separated after centrifugation and without any aqueous fractions, to determine bound 2- and 3-MCPD and bound glycidol is described later in section 4.6.3.2). The preparation of the aqueous sodium sulphate solution that contained the free analytes is presented in the following section 4.6.3.2).

4.6.3.1) Sample preparation of the polar fraction from compound foods to determine free 2- & 3-MCPD

The aqueous phase obtained from the fraction separation detailed previously in section *4.6.3)* was washed with 2.5 mL of a mixture of isohexane/tBME 4:1 (v,v) and the organic phase was then discarded. The aqueous phase was then extracted three times using 2 mL diethyl ether or a mixture of diethyl ether/ethyl acetate 9:1 (v,v) in each case. The organic phases were each separated following centrifugation using a Pasteur pipette, combined in an 8 mL screw cap vial with a spatula tip of sodium sulphate, 100 µL of phenylboronic acid solution (solution 7) added and concentrated to dryness under a stream of nitrogen. For the subsequent GC-MS measurement, the soluble residue was resorbed in 300 µL isooctane and a 200 µL aliquot was transferred to a GC insert. As particles frequently arose in the extracts when analysing heterogeneous compound foods, the aliquots intended for measurement were centrifuged and if sediment did arise, the clear supernatant was decanted into a new insert. Each analytical sequence included both a blank sample corresponding to the respective matrix and a reference sample with a known analyte content.

4.6.3.2) Sample preparation of the lipid fraction from compound foods to determine bound 2- and 3-MCPD and bound glycidol

The isohexane/tBME extracts obtained from the fraction separation detailed in section *4.6.3)* were combined in a weighed 8 mL screw cap vial and the solvent blown off in a stream of nitrogen. This process could be accelerated as needed by heating the base plate to 40 °C to 50 °C. The resulting fat phase was weighed out and by selectively separating excess materials or by adding a blank matrix (extra virgin olive oil), it was adjusted to 450 mg to 550 mg and fully dissolved by adding 3 mL tBME. The base-catalysed transesterification was then initiated by adding 1.4 mL of a methanolic sodium hydroxide solution (solution 1) at minus 26 °C. The reaction was terminated after 15 h to 18 h by adding 2.4 mL of an acidic stopping reagent (solution 2). If the transesterification was conducted over the weekend, diluted

reagent solutions (solution 2 and 4) were used accordingly; the reaction time then equated to 62 h to 66 h. To isolate the analytes, after removing the volatile solvent in the nitrogen stream, the reaction preparations were washed multiple times using 2 mL to 3 mL isohexane in each case or the same quantity of an isohexane/tBME mixture 4:1 (v,v). Pasteur pipettes were used to separate and discard the organic phases. The largely matrix-free aqueous phases were then extracted three times using 2 mL diethyl ether or a diethyl ether/ethyl acetate mixture in each case 9:1 (v,v). The organic phases were each separated following centrifugation using a Pasteur pipette, combined in an 8 mL screw cap vial with a spatula tip of sodium sulphate, 100 µL of phenylboronic acid solution (solution 7) added and concentrated to dryness under a stream of nitrogen. For the subsequent GC-MS measurement, the soluble residue was resorbed in 300 µL isooctane and a 200 µL aliquot was transferred to a GC insert. As particles frequently arose in the extracts when analysing heterogeneous compound foods, the aliquots intended for measurement were centrifuged and if sediment did arise, the clear supernatant was decanted into a new insert. Within each analytical sequence both a blank sample representing the matrix and a corresponding reference sample with a known analyte content were prepared. To determine the undesired transformation of 3-MCPD into glycidol, an analyte-free blank sample (the so-called transformation sample) was prepared for each analytical sequence. At the start of the work-up of the transformation sample, the standard working solution 3d was not used, instead 100 µL of each of the standard working solutions 5d and 6 (*d_5-3-MCPD-1,2-dioleoyl ester, 10 µg/mLd_5-3-MCPD equivalent in toluene and glycidyl oleate, 1 µg/mLglycidol equivalent in toluene*) was added.

4.7) GC-MS analysis

Essentially, the GC-MS analysis of the free and bound analytes was identical in terms of the used measurement system, the measurement program and the data evaluation as the bound MCPD and glycidyl derivatives were also carried over for the measurement in the free forms. The measurement conditions are published in detail [Kuhlmann, 2011; AOCS Cd 29b-13]. The only differences to the methods described were that, when evaluating the measurement of free 2- and 3-MCPD, signals for glycidyl derivatives were not included although this would have been technically possible. At this point, such an approach did not seem useful as the presence of free glycidol in foods is considered very unlikely and relevant analysis was not part of the reported project.

The data evaluation only differs from the conventional AOCS Cd 29b-13 (SGS "3-in-1" method) in its simplification that the raw glycidol values were not corrected for each sample individually but rather by determining representative transformation factors per analytical sequence [Kuhlmann, 2015]. In principle, all free or bound analytes were quantified via an internal 1-point calibration. Data analysis software, which was a component of the measurement system, was used for this purpose. The calculation stored in the software corresponded to the usual form of quantifying chromatographic analyte signals for the presence of signals of isotope-labelled internal standards with the same response characteristics.

$$\omega_{\text{Analyte A}} = \text{Peak area}_{\text{analyte A}} \cdot \omega_{\text{internal standard d5-A}} / \text{Peak area}_{\text{internal standard d5-A}}$$

Where:
ω = substance quantity (in mg/kg for the bound analytes and in µg/kg for the free analytes).
Analyte A: 2-MCPD or 3-MCPD or glycidol.

For the glycidol determination, this calculation gave a raw value that was then, using a transformation factor, reduced by the amount resulting from the proportional conversion of any 3-MCPD present in the sample at the same time into induced glycidol.

The transformation factor, as a measure of the proportion of induced glycidol resulting from 3-MCPD in the sample preparation, was determined for each analytical sequence from measuring the **transformation samples** using the following equations:

$$w_{inducedglycidol-d5} = \frac{PA_{inducedglycidol-d5} \times w_{glycidol-Tf}}{PA_{glycidol-Tf}}$$

Where:

	$w_{induced\ glycidol\text{-}d5}$	quantity of induced glycidol-d_5, in mg/kg
	$w_{glycidol\text{-}Tf}$	quantity of glycidol in Tf sample, in mg/kg
	$PA_{induced\ glycidol\text{-}d5}$	peak area of induced glycidol-d_5 (via 3-MBPD-d_5 - PBA derivative)
	$PA_{glycidol}$	peak area of glycidol in Tf sample (via 3-MBPD - PBA derivative)

$$Tf = \frac{w_{inducedglycidol-d5}}{w_{3-MCPD-d5}}$$

Where:	Tf	transformation factor
	$w_{induced\ glycidol\text{-}d5}$	quantity of induced glycidol-d_5, in mg/kg
	$w_{3\text{-}MCPD\text{-}d5}$	quantity of 3-MCPD-d_5, in mg/kg

The raw glycidol values from the food sample analyses were corrected using the following calculation:

$$\omega_{Glycidol} = \omega_{raw\ glycidol\ value} - (\omega_{3\text{-}MCPD} \cdot TF)$$

Where: ω: quantity in mg/kg Tf: transformation factor

4.8) Method validation

The methodology described in the previous sections was subject to in-house validation for various matrices. There was a particular focus on infant formula because these results were considered to be especially relevant. In addition, this matrix posed a problem in that a majority of the contained fats and thereby also the bound analytes are bound in a form being not accessible to normal extraction. This means it would have been useless to dope a blank material with the bound analytes for validation purposes because these would be too easy to extract in comparison with the compounds actually present in the samples. For this reason, the linearity of the method of the analysis was determined by mixing a contaminated retail product in defined proportions with an uncontaminated milk powder, which also served as a blank material. No recovery rates were determined for milk powder for this reason because the actual levels were unknown. By homogeneously dispersing the internal standard during HUPsS extraction and the preparation of the internal one-point calibration, and taking the results achieved in the past for other matrices into account, virtually quantitative recoveries can be expected. This was confirmed by multiple measurements of a reference material from a method validation study (see section 5.1). As free 2- and 3-MCPD is not usually present in milk powders and its molecular structure should make it easier to extract compared with the bound analytes, validation was performed by spiking. A worst-case scenario was also intendedly induced for other matrices whereby the samples with decreasing levels of bound

analytes were doped with increasingly higher quantities of free analytes. In this way, it could be ensured that there was no relevant carry-over of the free analytes to the bound analyte fraction.

The method validation for the bound analytes in oils and fats has already been published in detail [Kuhlmann, 2016]. The validation for the free analytes in the same matrix was separately achieved by escalating the doping of analyte-free glycerin with the reference substances. This matrix is not identical to pure oils and fats, however, it was prepared identically and better reflected samples that contain emulsifiers such as may be the case with margarines. This validation played a secondary role in practice because the free analytes are removed when refining oils and fats and therefore are not expected in these products. In fact, in the refined oils and comparable matrices studied as part of this project, no relevant quantities of free MCPD were detected.

To validate the compound foods with readily extractable oil and fat fractions, a shortbread biscuit blank matrix was used, which was doped with different concentrations of the free and bound analytes.

The limits of quantification were defined as the concentrations for which there was a signal-to-noise ratio ≥ of 9. To make it easier to analyse the raw data, and in particular, transfer the results and statistically evaluate the data, the matrix-related maximum limit of quantification for each of the bound analytes and the free compounds, and for all other matrices was set as the same value (10 µg/kg for each of the bound analytes, 5 µg/kg for each of the free analytes). The limits of detection, for which a signal-to-noise ratio ≥ of 3 was used as a basis, were predominantly set as 5 µg/kg for each of the bound analytes and 3 µg/kg for each of the free analytes. Although a consideration of the limits of detection including all outliers would have gone beyond the scope of this project, it was possible to record limits of detection actually achieved for the bound analytes at a factor of 2.5 below the original specifications, the limits of detection were therefore not considered further in the evaluation. The results presented in chapter 5 also show that this consideration had no significant influence on the overall results.

The key data produced from the method validations are presented as tables together with the overview of results in chapter 5 to enable direct association between the determined analyte concentrations and the key method data. The precision data also presented were not derived

from the method validation but from the quality assurance determinations performed in parallel to guarantee the timely connection to the measurements for this project.

4.9) Practical adaptations

The procedure described in the preceding sections was modified in certain cases. This was a necessary step, for example, when analyte concentrations emerged that were above the calibrated range. Repeated analyses were conducted in these cases in which the concentrations of the internal standard substances were increased (for example by using working solution 3d instead of 4d). Alternatively, samples with extremely high analyte contents were diluted with the blank matrix to reach a linear measurement range. For example, a typical method was to make 100 mg of a highly contaminated oil up to 1 g with analyte-free unrefined olive oil, to thoroughly homogenise the mixture by shaking and then analysing an aliquot of this mixture as a 10-fold dilution of the original sample in accordance with the process specification.
In principle, unusual findings such as the unexpected occurrence of analytes, were confirmed through repeat measurements. This could mean the detection of free MCPD in oils, fats or margarine samples or the occurrence of bound analytes in unrefined matrices.

5) Detailed expression of results

Essentially, the objective of this project is to provide data about the presence of 3-MCPD esters and related compounds in selected relevant food groups for the purposes of improved exposure assessment. As planned, at least 1050 food samples were tested for 5 different analytes, i.e. free 2-and 3-MCPD, bound 2- and 3-MCPD and bound glycidol. The resulting 5250 data points were evaluated according to statistical aspects for all investigated food groups. The results are presented and discussed in the following sections. In order to also evaluate the analytical results with regard to the analytical methods used, the data obtained from the ongoing quality assurance process and from the validation are also included in the individual sections. The number of positive results > LOQ, minimum and maximum values, 90th and 95th percentile (P90 and P95), and the mean and median values are listed for all food groups in addition to the total number of datasets and for each individual analyte. The latter values, as requested by the BfR, were calculated both on the basis of the lower-bound approach where all measurements below the limit of quantification are set to 0 and on the basis of the upper-bound approach where all measurements below the limit of quantification are set to the value of the limit of quantification. There was practically no difference between

the results from the upper-bound or lower-bound approach in datasets where no values or a negligibly low number of values lay below the limit of quantification. Just one value was reported here for reasons of improved clarity. All the analyte contents listed in the tables always refer to the food itself and not to the oil or fat content in compound foods. When evaluating the data, the underlying oil or fat content was estimated in individual cases from the analyte contents in compound products, this is however expressly indicated in each case. This procedure serves to identify highly polluted oils and fats that may have been used during food production or preparation. We define oils and fats with glycidol contents of over 1 mg/kg and 3-MCPD contents of over 2 mg/kg as highly contaminated.

5.1) Summary of results for infant formula

In total, the analytical results based on 221 samples of infant formula are reported. This included 119 samples of infant formula 1 and 102 samples of infant formula PRE. With regard to the products K-1, N-1, N-PRE, there were so few batch changes during the project that only 2 to 6 of the 10 batches could be sampled. The PRE foodstuff corresponding to the product H-1 was not available. The analyte contents determined in this product group are shown in Table 4:

Presence of 3-MCPD esters and related compounds in infant formula					
221 datasets	**Bound glycidol**	**Bound 3-MCPD**	**Bound 2-MCPD**	**Free 3-MCPD**	**Free 2-MCPD**
Measurements < LOQ, abs.	**122**	**0**	**4**	**221**	**221**
Measurements > LOQ, abs.	**99**	**221**	**217**	**0**	**0**
Measurements > LOQ, rel.	**45 %**	**100 %**	**98 %**	**0 %**	**0 %**
LOQ [µg/kg]	**10**	**10**	**10**	**5**	**5**
Minimum [µg/kg]	**< 10**	**20**	**< 10**	**< 5**	**< 5**
Median lb [µg/kg]	**0**	**103**	**38**	**NA**	**NA**
Median ub [µg/kg]	**10**	**103**	**38**	**NA**	**NA**
Mean lb [µg/kg]	**27**	**137**	**53**	**NA**	**NA**
Mean ub [µg/kg]	**32**	**137**	**54**	**NA**	**NA**
P90 [µg/kg]	**74**	**227**	**97**	**NA**	**NA**

P95 [µg/kg]	**154**	**270**	**108**	**NA**	**NA**
Max [µg/kg]	**536**	**1197**	**606**	**NA**	**NA**
Quality assurance data					
Reference A [µg/kg]	10	332	145	< 5	< 5
RSD, n = 19	12 %	3 %	3 %	NA	NA
Reference B [µg/kg]	25	331	146	< 5	< 5
RSD, n = 11	13 %	6 %	3 %	NA	NA
Blank A, n = 24	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Validation data for the infant formula matrix (milk powder)					
r^2	0.9978	0.9989	0.9984	0.9983	0.9983
c_v [µg/kg], n_v = 5-10	45-450	120-1200	60-600	2.5-45	2.5-45
r^2	0.9993	0.9994	0.9995	0.9981	0.9982
c_v [µg/kg], n_v = 10	5.5-55	9-90	2.8-28	50-275	50-275
rel.: relative; abs.: absolute; lb: lower bound; up: upper bound; NA.: not applicable					
RSD: Relative standard deviation, r^2: Correlation coefficient, c_v: Concentration range of validation; n_v: Number of concentration levels					

Table 4: Summary of results for infant formula

The analysis of all investigated infant formula products was implemented without problems. There were no interferences or unusual measurement results, and it was not necessary to raise the limits of quantification in any cases. Two measures of infant formula originating from the BfR method validation for the determination of MCPD fatty acid esters in fatty foods were used as reference samples [Fry et al.: 2013]. These measures only differ with regard to their content of bound glycidol. The availability of this reference material must be seen as unique as no other official method validations using this matrix or other compound foods have been published to date. The values determined by BfR for the reference material P1 at the time were 1.30 mg/kg bound 3-MCPD and 0.64 mg/kg bound 2-MCPD, each relating to the fat phase and with a fat content of 24.5 %; the glycidol contents were not determined [Fry et al.: 2013]. Based on the total sample, the calculation gave rise to an expected value of 319 µg/kg 3-MCPD and 157 µg/kg 2-MCPD. These values can of course only serve as rough orientation due to the method uncertainty at the time and the long storage period of the samples. However, the agreement of the measured values with the expected values determined during

this study can be seen as very satisfactory and evidences good comparability of the results which have been determined with very different methods. The lack of free 3-MCPD in the reference material did not play a role as this also applied to all investigated milk powder samples. The relative standard deviations derived from the current processing of the reference samples for bound 2- and 3-MCPD were each very low in the single digit range (3 % - 6 %), even though the measurement period covered several months and analysis was carried out by various operators. The determination of the reference glycidol contents just above or on the limit of quantification showed a large variation (RSD = 12 % - 13 %), which was presumably not caused only by the increasing measurement uncertainty at lower concentrations, but also by the effect, as already described in the introduction, that the result uncertainty for low glycidol contents increases during alkaline transesterification if the 3-MCPD values are high. Blank A is a largely analyte-free milk powder of foreign origin. The precise source and trade name are unknown.

The investigated infant formula products to some extent display significant variance with regard to analyte distribution. Free MCPD above the limit of quantification could not be found in any samples. This meets expectations as the presence of the free compounds was not probable due to the normal ingredients or the production conditions during infant formula production. In contrast, the presence of bound 2- and 3-MCPD in infant formula was practically ubiquitous, just 4 of the 221 samples evidenced 2-MCPD just below the limit of quantification. However, the values for the 90th and 95th percentiles with regard to the mean and median values show that the variation of bound MCPD content in infant formula is lower than in other foodstuffs, which indicates stricter control of production conditions or stricter selection of the raw products. The situation regarding the presence of bound glycidol in infant formula was different. The content in the majority of samples was below 10 µg/kg, the limit of quantification for the method. Generally, the measured glycidol concentrations lay several factors or even orders of magnitude below that for the 3-MCPD occurring in parallel. With this large portion of findings < LOQ for glycidol, the relevant question is whether the median values in particular were determined using a lower-bound approach or upper-bound approach. Both approaches were considered comparatively in the evaluation. To answer the question as to which scenario is more realistic, it was estimated what order of magnitude the signals display under the limit of quantification. In fact, a relatively symmetrical distribution of values just below the limit of quantification right down to a complete lack of signals was found. From this perspective, a middle-bound calculation would be justified here. If one takes

into consideration that the bound glycidol at contents > 17 µg/kg falls below the MoE of 10000, as presented in the introduction [BfR: 2009], which applies for the period of data collection, this was not the case for the majority of infant formula products found on the market. When looking at how far the contents of the bound analytes correlate, it is evident that the ratio between both MCPD isomers was very stable across all samples. 2-MCPD contributes on average to approx. 27 % of the total MCPD content in a sample (RSD 20 %). This is a typical ratio which can be found in refined oils and fats that are not subjected to extreme deodorisation temperatures [Kuhlmann: 2016]. In contrast, the glycidol contents do not correlate much with the MCPD values. However, a rough trend was identified that products with particularly high concentrations of bound glycidol also show on average increased MCPD contents. For this reason, any evaluation of whether the highest analyte contents (> P90) can be assigned to specific manufacturers or production periods must differentiate between analytes. A clear dependency on two manufacturers was noted for the high glycidol contents, whereby in one case all batches had a best before date (BBD) in 2016, while newer batches with a BBD in 2017 contained significantly lower glycidol quantities. The assignment of specific manufacturers or production periods regarding the highest determined MCPD contents was less indicative. The manufacturer whose products contained the highest glycidol contents was also represented in over 50 % of the samples where the bound MCPD exceeded the 90th percentile.

5.2) Summary of results for refined vegetable oils (edible oils)

180 edible vegetable oils were tested in accordance with the sampling plan. There were no analytical problems. Refined rapeseed and sunflower seed oil accounted for the majority with 50 and 58 samples each; these have a high market share in Germany. 20 olive oils and 10 corn oils were also tested as common oil types. 42 samples consisted mainly of various seed and fruit oils (in descending order: sesame, peanut, grapeseed, safflower, hazelnut, walnut, linseed, soya, palm, wheat germ, pumpkin seed). Depending on market availability, organic products were included in the investigation in addition to conventional goods. Table 2 shows the exact sample key. Linseed and pumpkin seed oils could only be obtained in an unrefined form. According to our current knowledge, these oils in their pure form are normally sold unrefined as otherwise their taste and the valuable ingredients would be lost. The results shown in Table 5 therefore do not refer solely to refined oils, but also to unrefined products in 5 cases.

Presence of 3-MCPD esters and related compounds in vegetable oils (edible oils)					
180 datasets	Bound glycidol	Bound 3-MCPD	Bound 2-MCPD	Free 3-MCPD	Free 2-MCPD
Measurements < LOQ, abs.	5	4	9	178	180
Measurements > LOQ, abs.	175	176	171	2	0
Measurements > LOQ, rel.	97%	98%	95%	1%	0%
LOQ [µg/kg]	10	10	10	5	5
Minimum [µg/kg]	< 10	< 10	< 10	< 5	< 5
Median lb = ub [µg/kg]	291	297	125	< 5	NA
Mean lb = ub [µg/kg]	396	609	274	< 5	NA
P90 [µg/kg]	862	1213	552	< 5	NA
P95 [µg/kg]	1340	1556	680	< 5	NA
Max [µg/kg]	1798	20935	9770	11	NA
Quality assurance data					
Reference C [µg/kg]	637	522	226	< 5	< 5
RSD, n = 42	2%	2%	3%	NA	NA
Reference D [µg/kg]	< 10	< 10	< 10	9.4	9.1
RSD, n = 24	NA	NA	NA	6%	5%
Reference E [µg/kg]	< 10	< 10	< 10	5.2	5.4
RSD, n = 10	NA	NA	NA	6%	7%
Blank C, n = 42	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
rel.: relative; abs.: absolute; lb: lower bound; up: upper bound; NA.: not applicable					

Validation data for the oils, fats, margarine matrix					
r^2	0.9999	0.9999	0.9998		
c_v [µg/kg], n_v = 8	10 - 100	10 - 100	10 - 100		
Recovery, rel. [%]	99 +/- 2	98 +/- 4	100 +/- 2		
r^2	0.9984	0.9997	0.9999	0.9991	0.9988
c_v [µg/kg], n_v = 7/9	100 - 2000	100 - 2000	100 - 2000	50	1000
Recovery, rel. [%]	99 +/- 3	99 +/- 2	99 +/- 2	96 +/- 7	98 +/- 3
rel.: relative; abs.: absolute; lb: lower bound; up: upper bound; NA.: not applicable					
RSD: Relative standard deviation, r^2: Correlation coefficient, c_v: Concentration range of validation; n_v: Number of concentration levels					

Table 5: Summary of results of plant oils (edible oils)

The tabulated summary shows that bound analytes were found above the limit of quantification in all refined oils. Bound 3-MCPD was present with values > 10 µg/kg in all products that were fully or partially refined, as well as in one unrefined pumpkin seed oil. Bound glycidol and bound 2-MCPD were found just under the limit of quantification in various rapeseed oils. *Vice versa* and as was expected, the bound contaminants were not found in the unrefined oils. One exception was the roasted pumpkin seed oil where 11 µg/kg bound 3-MCPD was detected. The roasting process is seen as the most obvious cause for this result.

The edible oils represent a product with very different content levels, particularly with regard to bound 3-MCPD. In some cases, certain oils, such as e.g. rapeseed oil, have a relatively low level on average, while other types indicate significantly higher analyte levels in individual cases. This is expressed in a large difference between the median and average values. The values for bound glycidol were relatively low as expected in the product group investigated here – e.g. in comparison to refined palm oil or palm fat. More than 80% of the results lay below a value of 1 mg/kg for bound glycidol.

The free analytes as expected only play a marginal role in edible oils. It is assumed that these compounds are fully removed during refining due to their volatility. Migration from packaging material, usually glass, is also improbable. The 2 positive results for free 3-MCPD occurred in this product group, in the hazelnut oil with the highest level and in a roasted sesame oil. In the first case, it can be assumed that, if there is a content in excess of 20 mg/kg

of bound 3-MCPD, minimal natural decomposition reactions will release small quantities of the core analyte. 11 µg/kg free 3-MCPD was found. The presence of approximately 7 µg/kg free 3-MCPD in a roasted sesame oil presupposes the assumption that the roasting process is the probable source for the occurrence of the free analyte. Signals for free 3-MCPD below the limit of quantification also occurred regularly in other roasted oils. The argument that the free analytes in roasted oils are completely removed during refinement is only partially applicable as many oils consist of a combination of refined and unrefined components. The latter are intended to transmit the typical taste and odour of the product.

The reference material C consists of a mixture of refined rapeseed oil and refined palm oil with a representative concentration of bound analytes. The modified "3-in-1" analytic method used for the matrix group oils, fats, margarines was shown as very robust which is expressed in the remarkably low standard deviations of the reference measurements. As there are currently practically no oils available that contain both free analytes above the limit of quantification, analyte-free olive oil (extra virgin) was doped with free 2-MCPD and free 3-MCPD to produce reference materials D and E. Very low concentrations were deliberately selected at double the limit of quantification and at the limit of quantification in order to depict the concentrations to be expected in reality. The same olive oil (extra virgin) without any doping was used as the blank material C. Two cases within the 42 blank measurements were detected with a bound analyte just above the limit of quantification. The cause here is assumed to be minimal carry-over following the processing of highly loaded samples. This cannot always be completely avoided if the concentration differences between the limit of quantification and the maximum contents are extremely large (for example, with around 3-MCPD, the highest measured quantity was more than 2000 times above the LOQ). However, as shown by the constancy of the results for the reference samples, the effects do not play a role in practice as the analyte content in all refined oils and fats were several orders of magnitude above the limit of quantification. In contrast, during the analysis of unrefined samples with no or low loads, no significant carry-over was noted so that no limitations with regards precision and accuracy of the results were seen for these measurements.

5.2.1 Rapeseed oils

In order to assess more clearly whether the various oil types contribute on average differently to the level of 3-MCPD esters and related compounds, the evaluation described above was broken down and applied in an identical manner to the individual oil types. Tables 6 to 10 set

out a summary of the results. The data on analytical quality assurance are not listed again as they are identical to those in Table 5.

Presence of 3-MCPD esters and related compounds in refined rapeseed oils					
50 datasets	**Bound glycidol**	**Bound 3-MCPD**	**Bound 2-MCPD**	**Free 3-MCPD**	**Free 2-MCPD**
Measurements < LOQ, abs.	**0**	**0**	**0**	**50**	**50**
Measurements > LOQ, abs.	**50**	**50**	**46**	**0**	**0**
Measurements > LOQ, rel.	**100%**	**100%**	**92%**	**0%**	**0%**
LOQ [µg/kg]	**10**	**10**	**10**	**5**	**5**
Minimum [µg/kg]	**32**	**16**	**< 10**	**NA**	**NA**
Median lb = ub [µg/kg]	**157**	**70**	**25**	**NA**	**NA**
Mean lb = ub [µg/kg]	**176**	**172**	**69**	**NA**	**NA**
P90 [µg/kg]	**306**	**403**	**181**	**NA**	**NA**
P95 [µg/kg]	**342**	**507**	**246**	**NA**	**NA**
Max [µg/kg]	**350**	**1684**	**672**	**NA**	**NA**
rel.: relative; abs.: absolute; lb: lower bound; up: upper bound; NA.: not applicable					
Quality assurance data: identical to Table 5					

Table 6: Summary of results for refined rapeseed oils

Results regarding the presence of 3-MCPD esters and related compounds in refined rapeseed oils indicate that, with regard to the presence of the bound analytes, the majority of the investigated samples have comparable low levels of bound 2- and 3-MCPD in particular. They therefore differ to some extent from the other investigated oils and fats, including those in other product groups. Bound MCPD was found in the lower µg/kg range and even the 2-isomer was found to be just under the limit of quantification in a significant number of samples. The levels of bound glycidol were also lower on average than in the other investigated oil types. Clear differences between conventional and organic products could not be ascertained. On the one hand, several organic rapeseed oils were amongst the products with the lowest levels, but on the other hand, the highest determined levels of 2- and 3-MCPD

were found in an organic product. None of the investigated samples showed levels of more than 2 mg/kg 3-MCPD or 1 mg/kg glycidol.

5.2.2 Sunflower oils

The contamination situation for the investigated refined sunflower oils showed, in comparison to the rapeseed oils, higher levels of bound analytes on average. The value distribution between minimum and maximum values was also more homogeneous with respect to the bound MCPDs so that the relative difference between median and mean values was lower. There is therefore apparently a series of sunflower oils with relatively low levels, but at the same time proportionally more products with medium and high levels of contamination. In the case of MCPD, none of the 58 samples exceeded a level of 2 mg/kg, however, the series did contain 3 samples where values for bound glycidol exceeded 1 mg/kg. A summary of results is shown in Table7a.

Presence of 3-MCPD esters and related compounds in refined sunflower oils (conventional and organic products)					
58 datasets	**Bound glycidol**	**Bound 3-MCPD**	**Bound 2-MCPD**	**Free 3-MCPD**	**Free 2-MCPD**
Measurements < LOQ, abs.	**0**	**0**	**0**	**58**	**58**
Measurements > LOQ, abs.	**58**	**58**	**58**	**0**	**0**
Measurements > LOQ, rel.	**100 %**	**100 %**	**100 %**	**0 %**	**0 %**
LOQ [µg/kg]	**10**	**10**	**10**	**5**	**5**
Minimum [µg/kg]	**93**	**36**	**13**	**NA**	**NA**
Median lb = ub [µg/kg]	**299**	**324**	**129**	**NA**	**NA**
Mean lb = ub [µg/kg]	**412**	**376**	**176**	**NA**	**NA**
P90 [µg/kg]	**769**	**726**	**351**	**NA**	**NA**
P95 [µg/kg]	**955**	**820**	**395**	**NA**	**NA**
Max [µg/kg]	**1798**	**1197**	**577**	**NA**	**NA**
rel.: relative; abs.: absolute; lb: lower bound; up: upper bound; NA.: not applicable					
Quality assurance data: identical to Table 5					

Table 7a: Summary of results for refined sunflower oils (conventional and organic products)

In the case of sunflower oils, a significant difference was noted between the average results for conventional and organic products. Even though, as listed in Table 7b, the lowest findings came from an organic sunflower oil, however for the bound glycidol levels in particular, all the other key data was higher than in the complete sample set.

Presence of 3-MCPD esters and related compounds in refined sunflower oils (organic products)					
18 datasets	**Bound glycidol**	**Bound 3-MCPD**	**Bound 2-MCPD**	**Free 3-MCPD**	**Free 2-MCPD**
Measurements < LOQ, abs.	**0**	**0**	**0**	**18**	**18**
Measurements > LOQ, abs.	**18**	**18**	**18**	**0**	**0**
Measurements > LOQ, rel.	**100 %**	**100 %**	**100 %**	**0 %**	**0 %**
LOQ [µg/kg]	**10**	**10**	**10**	**5**	**5**
Minimum [µg/kg]	**93**	**36**	**13**	**NA**	**NA**
Median lb = ub [µg/kg]	**370**	**302**	**119**	**NA**	**NA**
Mean lb = ub [µg/kg]	**480**	**316**	**133**	**NA**	**NA**
P90 [µg/kg]	**966**	**489**	**220**	**NA**	**NA**
P95 [µg/kg]	**1297**	**505**	**238**	**NA**	**NA**
Max [µg/kg]	**1798**	**594**	**270**	**NA**	**NA**
rel.: relative; abs.: absolute; lb: lower bound; up: upper bound; NA.: not applicable					
Quality assurance data: identical to Table 5					

Table 7b Summary of results for refined sunflower oils (organic products)

5.2.3 Olive oils

The following summary of results, based on a relatively small number of analyses, for the contamination level in olive oils is characterised by one sample in which no analytes were found. The product was not marked as virgin or refined, so it can be assumed that this sample is an unrefined product. For this reason, Table 8 also lists the P05 value, which indicates the lowest found value range for refined olive oils.

Presence of 3-MCPD esters and related compounds in olive oils					
20 datasets	**Bound glycidol**	**Bound 3-MCPD**	**Bound 2-MCPD**	**Free 3-MCPD**	**Free 2-MCPD**
Measurements < LOQ, abs.	**1**	**1**	**1**	**20**	**20**
Measurements > LOQ, abs.	**19**	**19**	**19**	**0**	**0**
Measurements > LOQ, rel.	**95 %**	**95 %**	**95 %**	**0 %**	**0 %**
LOQ [µg/kg]	**10**	**10**	**10**	**5**	**5**
Minimum [µg/kg]	**< 10**	**< 10**	**< 10**	**NA**	**NA**
P05 [µg/kg]	**90**	**168**	**58**	**NA**	**NA**
Median lb = ub [µg/kg]	**307**	**385**	**167**	**NA**	**NA**
Mean lb = ub [µg/kg]	**428**	**395**	**171**	**NA**	**NA**
P90 [µg/kg]	**811**	**648**	**249**	**NA**	**NA**
P95 [µg/kg]	**1344**	**659**	**309**	**NA**	**NA**
Max [µg/kg]	**1420**	**713**	**333**	**NA**	**NA**
rel.: relative; abs.: absolute; lb: lower bound; up: upper bound; NA.: not applicable					
Quality assurance data: identical to Table 5					

Table 8: Summary of results for olive oils

The data showed that the levels of MCPD and glycidyl esters were roughly comparable to those found in refined sunflower oils. Fruit oils tend more strongly towards thermally induced formation of 3-MCPD esters and related compounds, so that the levels detected appear relatively low compared to expectations. However, it must be considered here that fully refined olive oils are probably not available in the German retail market, instead only those that contain a certain percentage of virgin olive oil for sensory reasons. The actual percentage does not need to be declared, so that the actual analyte contents in the refined oil remain unknown.

5.2.4 Corn oils

With just 10 samples, the corn oils represent the smallest separately evaluated edible oil type in this study. The results are summarised in Table 9.

Presence of 3-MCPD esters and related compounds in refined corn oils					
10 datasets	**Bound glycidol**	**Bound 3-MCPD**	**Bound 2-MCPD**	**Free 3-MCPD**	**Free 2-MCPD**
Measurements < LOQ, abs.	**0**	**0**	**0**	**10**	**10**
Measurements > LOQ, abs.	**10**	**10**	**10**	**0**	**0**
Measurements > LOQ, rel.	**100 %**	**100 %**	**100 %**	**0 %**	**0 %**
LOQ [µg/kg]	**10**	**10**	**10**	**5**	**5**
Minimum [µg/kg]	**560**	**452**	**207**	**NA**	**NA**
Median lb = ub [µg/kg]	**726**	**1081**	**518**	**NA**	**NA**
Mean lb = ub [µg/kg]	**871**	**1052**	**505**	**NA**	**NA**
P90 [µg/kg]	**1418**	**1440**	**669**	**NA**	**NA**
P95 [µg/kg]	**1445**	**1495**	**750**	**NA**	**NA**
Max [µg/kg]	**1473**	**1549**	**830**	**NA**	**NA**
rel.: relative; abs.: absolute; lb: lower bound; up: upper bound; NA.: not applicable					
Quality assurance data: identical to Table 5					

Table 9: Summary of results for refined corn oils

In summary, corn oils present a higher level of contamination compared to the refined edible oils discussed above. It is particularly noticable that there were no samples with low levels. However, these results may be representative of the exposure due to corn oils, but must be viewed as less representative of different products as 9 of the 10 samples purchased proportionally to the market shares originated from one manufacturer.

5.2.5 Other oils

The other oils evaluated within this project include various edible oils, some of which are counted as speciality oils. These include varieties such as linseed oil or pumpkin seed oil which are commercially available unrefined. The results obtained were still included in the evaluation as they clearly prove that the formation of 3-MCPD esters and related compounds is thermally induced. The summary of results in Table 10 shows a very heterogeneous picture, as expected, regarding the presence and distribution of the analytes.

Presence of 3-MCPD esters and related compounds in other oils					
42 datasets	**Bound glycidol**	**Bound 3-MCPD**	**Bound 2-MCPD**	**Free 3-MCPD**	**Free 2-MCPD**
Measurements < LOQ, abs.	**4**	**3**	**4**	**40**	**42**
Measurements > LOQ, abs.	**38**	**39**	**38**	**2**	**0**
Measurements > LOQ, rel.	**90 %**	**93 %**	**90 %**	**5 %**	**0 %**
LOQ [µg/kg]	**10**	**10**	**10**	**5**	**5**
Minimum [µg/kg]	**< 10**	**< 10**	**< 10**	**NA**	**NA**
Median lb = ub [µg/kg]	**425**	**573**	**189**	**NA**	**NA**
Mean lb = ub [µg/kg]	**509**	**1447**	**646**	**NA**	**NA**
P90 [µg/kg]	**1218**	**2915**	**1418**	**NA**	**NA**
P95 [µg/kg]	**1355**	**3516**	**1617**	**NA**	**NA**
Max [µg/kg]	**1597**	**20935**	**9770**	**11**	**NA**
rel.: relative; abs.: absolute; lb: lower bound; up: upper bound; NA.: not applicable					
Quality assurance data: identical to Table 5					

Table 10: Summary of results for other oils

It can be presumed that manufacturer are less aware of the formation of MCPD and glycidol during refining for more rarely used edible oils than those that are mass produced. It is possible that many of the investigated products are produced by smaller companies or refineries that are not technically state of the art. This could be the reason why individual

samples in this product group of “speciality oils” evidence very high analyte levels even though this is not the case for the other tested oils of the same type.

This group of “speciality oils” therefore contains various oils where 3-MCPD > 2 mg/kg and glycidol > 1 mg/kg were found, corresponding to a relatively high level of contamination. It is noticeable that the maximum value found in a hazelnut oil for bound 3-MCPD is, at over 20 mg/kg, around 3 orders of magnitude higher than more weakly contaminated refined oils. This value can assuredly be treated as an outlier, compared to the other significantly lower key data. The other two hazelnut oils that were tested also showed much lower levels of contamination at approx. 1.4 mg/kg and 1.0 mg/kg. In comparison, nut oils with very high contents of bound 3-MCPD were in part previously reported [Kuhlmann, 2011]. In order to ascertain whether this detected peak level was actually an outlier with regard to the product line, a second batch of the same product from the same manufacturer was tested. The bound 3-MCPD levels found here were even higher with over 50 mg/kg and over 25 mg/kg for bound 2-MCPD. These values were not included in the statistical evaluation, but they do show that there are apparently even greater differences in contamination levels regarding 3-MCPD and related compounds in nut oils.

5.3) Summary of results for margarine

Margarine is certainly one of the foodstuffs produced in very high volumes that contain significant quantities of refined oils and fats. The investigation for the presence of 3-MCPD esters and related compounds in the largest possible number of samples was therefore necessary to obtain the most realistic exposure assessment for this foodstuff. The values listed in Table 11 indicate that the three bound analytes occur in parallel and ubiquitously in this foodstuff group.

Presence of 3-MCPD esters and related compounds in margarine					
200 datasets	**Bound glycidol**	**Bound 3-MCPD**	**Bound 2-MCPD**	**Free 3-MCPD**	**Free 2-MCPD**
Measurements < LOQ, abs.	**0**	**0**	**0**	**194**	**200**
Measurements > LOQ, abs.	**200**	**200**	**200**	**6**	**0**
Measurements > LOQ, rel.	**100 %**	**100 %**	**100 %**	**3 %**	**0 %**
LOQ [µg/kg]	**10**	**10**	**10**	**5**	**5**
Minimum [µg/kg]	**14**	**23**	**12**	**< 5**	**NA**
Median lb = ub [µg/kg]	**204**	**384**	**180**	**< 5**	**NA**
Mean lb = ub [µg/kg]	**224**	**398**	**188**	**< 5**	**NA**
P90 [µg/kg]	**357**	**675**	**324**	**NA**	**NA**
P95 [µg/kg]	**381**	**697**	**340**	**NA**	**NA**
Max [µg/kg]	**1100**	**1043**	**497**	**44**	**NA**
rel.: relative; abs.: absolute; lb: lower bound; up: upper bound; NA.: not applicable					
Quality assurance data: identical to Table 5					

Table 11: Summary of results for margarine

In fact in none of the 200 samples one of the ester-bound contaminants could not be found. The detected analyte contents were distributed relatively symmetrically, so that median and mean values were relatively close to each other; just over or under 200 µg/kg for glycidol and 2-MCPD and just under 400 µg/kg for 3-MCPD. In terms of magnitude, the results were therefore in a range that occurs in numerous edible oils and which, with regard to the relatively large number of samples, evidence very few upward outliers. One reason for this is that margarine is usually produced by large manufacturers who presumably take into account the topic of 3-MCPD and glycidol in this matrix and who may have established good process control, or who use oils and fats that are not highly contaminated. This point must be looked at in future from a more differentiated aspect as the fat content in the whole product group

varies strongly between 25 % and 75 %, which means that the analyte contents increase proportionately in relation to the fat content.

The occasional presence of free 3-MCPD in this foodstuff group was not actually expected. The source remains unclear, although it is feasible that it is introduced via auxiliaries and additives during production, or via migration from the packaging material which generally always consisted of plastic. The determined levels of free 3-MCPD only contributed marginally however to the total 3-MCPD contamination in margarine, even though the highest determined value for free 3-MCPD exceeded the lowest value for bound 3-MCPD.

5.4) Summary of results for frying fats

As expected no samples were free of bound analytes in the frying fats, as shown in Table 12:

Presence of 3-MCPD esters and related compounds in frying fats					
50 datasets	**Bound glycidol**	**Bound 3-MCPD**	**Bound 2-MCPD**	**Free 3-MCPD**	**Free 2-MCPD**
Measurements < LOQ, abs.	**0**	**0**	**0**	**50**	**50**
Measurements > LOQ, abs.	**50**	**50**	**50**	**0**	**0**
Measurements > LOQ, rel.	**100 %**	**100 %**	**100 %**	**0 %**	**0 %**
LOQ [µg/kg]	**10**	**10**	**10**	**5**	**5**
Minimum [µg/kg]	**17**	**147**	**69**	**NA**	**NA**
Median lb = ub [µg/kg]	**1006**	**1570**	**706**	**NA**	**NA**
Mean lb = ub [µg/kg]	**1481**	**1927**	**756**	**NA**	**NA**
P90 [µg/kg]	**3925**	**2320**	**1154**	**NA**	**NA**
P95 [µg/kg]	**4443**	**2861**	**1385**	**NA**	**NA**
Max [µg/kg]	**8698**	**3408**	**1538**	**NA**	**NA**
rel.: relative; abs.: absolute; lb: lower bound; up: upper bound; NA.: not applicable					
Quality assurance data: identical to Table 5					

Table 12: Summary of results for frying fats

Frying fats represented the food group with the highest level of glycidol and MCPD fatty acid esters in this project, which means that the recorded minimum values should probably be viewed as outliers compared to the maximum values. The on average very high content of bound analytes can be most likely be explained by the significant proportion of products that consist of classic refined palm fat. It is remarkable that more than half of all samples evidenced bound glycidol levels of more than 1 mg/kg and more than one quarter of all samples evidenced 3-MCPD levels of over 2 mg/kg. Free MCPD did not play a role in this product group.

5.5) Summary of results for French fries

French fries are foods that could certainly be under suspicion of containing relevant quantities of process contaminants because they are produced and prepared using refined oils and fats. However, the expectation here is that the pre-fried frozen products should only be weakly contaminated with 3-MCPD esters and related compounds. The reasoning here is that industrial pre-frying is usually carried out in oils with low contamination levels, such as rapeseed oil, and that the fat content of the products only increases to approximately 10 % through this method. Based on the analyte contents in rapeseed oils shown in Table 6, it was expected that the MCPD and glycidol contents in frozen French fries would be close to the limit of detection. In contrast to fish and meat products, it is not normally expected that pre-frying vegetable foodstuffs produces additional MCPD and glycidyl esters. Based on these assumptions, the number of frozen French fries to be tested was reduced during the project phase and replaced by the purchase and analysis of ready-to-eat French fries. Consequently, the results were therefore separated into Table 13a for frozen products and 13b for ready-to-eat French fries purchased from takeaway outlets:

Presence of 3-MCPD esters and related compounds in pre-fried frozen French fries					
33 datasets	**Bound glycidol**	**Bound 3-MCPD**	**Bound 2-MCPD**	**Free 3-MCPD**	**Free 2-MCPD**
Measurements < LOQ, abs.	**11**	**4**	**17**	**33**	**33**
Measurements > LOQ, abs.	**22**	**29**	**16**	**0**	**0**
Measurements > LOQ, rel.	**67 %**	**88 %**	**48 %**	**0 %**	**0 %**
LOQ [µg/kg]	**10**	**10**	**10**	**5**	**5**
Minimum [µg/kg]	**< 10**	**< 10**	**< 10**	**NA**	**NA**
Median lb [µg/kg]	**12**	**16**	**0**	**NA**	**NA**
Median ub [µg/kg]	**12**	**16**	**10**	**NA**	**NA**
Mean lb [µg/kg]	**10**	**16**	**6**	**NA**	**NA**
Mean ub [µg/kg]	**13**	**17**	**11**	**NA**	**NA**
P90 [µg/kg]	**19**	**22**	**13**	**NA**	**NA**
P95 [µg/kg]	**21**	**26**	**13**	**NA**	**NA**
Max [µg/kg]	**22**	**35**	**13**	**NA**	**NA**
rel.: relative; abs.: absolute; lb: lower bound; up: upper bound; NA.: not applicable					
Quality assurance data: Section 5.10)					

Table 13a: Summary of results for pre-fried frozen French fries

In actual fact, the determined content of 3-MCPD esters and related compounds corresponded surprisingly well to the expected values. In some samples, the analyte content was below the limit of quantification; the maximum values were found to be in the lower two digits µg/kg range. Free MCPD did not play a role. There were no analytical issues, limits of quantification did not need to be increased.

It was assumed, with regard to ready-to-eat French fries, that a significant introduction of MCPD and glycidyl esters would occur due to frying. As a rough approximation, it can be assumed, although the fat content in the French fries does not increase significantly due to

frying and is usually not expected to be much over approx. 15 %, that the oil used during pre-frying is then replaced by the actual frying fat. Taking into account the values listed in Table 12 for frying fats, significantly higher analyte concentrations were expected compared to frozen products:

Presence of 3-MCPD esters and related compounds in ready-to-eat French fries (takeaway French fries)					
67 datasets	**Bound glycidol**	**Bound 3-MCPD**	**Bound 2-MCPD**	**Free 3-MCPD**	**Free 2-MCPD**
Measurements < LOQ, abs.	**3**	**1**	**1**	**32**	**51**
Measurements > LOQ, abs.	**64**	**66**	**66**	**35**	**16**
Measurements > LOQ, rel.	**96 %**	**99 %**	**99 %**	**52 %**	**24 %**
LOQ [µg/kg]	**10**	**10**	**10**	**5**	**5**
Minimum [µg/kg]	**< 10**	**< 10**	**< 10**	**< 5**	**< 5**
Median lb = ub [µg/kg]	**82**	**90**	**51**	**6**	**5**
Mean lb = ub [µg/kg]	**118**	**111**	**68**	**26**	**9**
P90 [µg/kg]	**250**	**231**	**142**	**42**	**17**
P95 [µg/kg]	**347**	**400**	**206**	**110**	**51**
Max [µg/kg]	**588**	**562**	**288**	**617**	**272**
rel.: relative; abs.: absolute; lb: lower bound; up: upper bound; NA.: not applicable					
Quality assurance data: Section 5.10)					

Table 13b: Summary of results for ready-to-eat French fries (takeaway French fries)

The broken down values regarding the presence of 3-MCPD esters and related compounds in ready-to-eat French fries shown in the above Table 13b show a very broad analyte distribution. One or the other bound analyte was found to be just under the limit of detection in very few samples. In general, all MCPD esters and the glycidyl esters appeared in association. The mean and median values lay approximately a factor of 5 to 7 higher than for

the pre-fried products and, in contrast to the frozen French fries, a series of samples were found whose analyte content exceeded the mean value several times. If the fat content of the samples is estimated to be approximately 15 %, the contamination of frying fats with bound glycidol and bound 3-MCPD should be calculated to be over 3 mg/kg respectively, which would be a typical order of magnitude for the series of more contaminated roasting and frying fats.

The presence of free 2- and 3-MCPD in the ready-to-eat French fries is especially notable in comparison to all other food groups that have been presented so far. It was ascertained that more than half of all samples contained free 3-MCPD, while a quarter of all samples also contained free 2-MCPD which, although this had not previously appeared relevant, was above the limit of quantification. In addition – and this was not expected – the measured contents of free MCPD were present in relevant orders of magnitude and even exceeded the amount of bound 3-MCPD occurring in parallel in the samples, so that the total MCPD content was significantly increased by the presence of the free analyte in some samples. It is unclear what the source of the free analytes could be during frying. On the one hand, it is feasible that chloride and C3 parent compounds are formed independently in the deep-fat fryers. On the other hand, it is possible that they are released from the bound analytes if one considers that a large number of different food components enter the fryers over a long period of frying, making a very complex number of chemical reactions therefore possible. The presumption that, in the case of frying, no free MCPD is likely to be introduced from the outside by the addition of auxiliary materials unknown to us, instead there is de-novo formation or a release from the bound form due to unknown chemical reactions in the deep-fat fryers. This presumption is supported by observing the ratios of 3-MCPD to 2-MCPD. While in practically all results shown here so far, the bound 2-MCPD had a share of approximately 25 to 35 % of the total content of MCPD esters, the proportion of free 2-MCPD in the total content of free MCPD was significantly lower in the case of the free isomers. These easily reproducible ratios were no longer constant in the case of the ready-to-eat French fries. To some extent, much higher percentages of 2-MCPD were found in both the bound and the free MCPD isomers. The 2-MCPD contents were actually higher than those of the 3-MCPD in a series of samples.

A separate evaluation, in which the results of free and bound 2-and 3-MCPD were summed up for each sample, was carried out to evaluate the total contamination of free and bound 2-

MCPD and 3-MCPD respectively. The results are shown in Table 13c. The limits of quantification for free MCPD were increased to 10 µg/kg to simplify evaluation.

Results for total MCPD content in ready-to-eat French fries (takeaway)		
67 datasets	**Total 3-MCPD**	**Total 2-MCPD**
Measurements < LOQ, abs.	**1**	**1**
Measurements > LOQ, abs.	**66**	**66**
Measurements > LOQ, rel.	**99 %**	**99 %**
LOQ [µg/kg]	**10**	**10**
Minimum [µg/kg]	**< 10**	**< 10**
Median lb [µg/kg]	**91**	**54**
Median ub [µg/kg]	**91**	**54**
Mean lb [µg/kg]	**138**	**78**
Mean ub [µg/kg]	**138**	**78**
P90 [µg/kg]	**488**	**237**
P95 [µg/kg]	**620**	**285**
Max [µg/kg]	**1102**	**484**
rel.: relative; abs.: absolute; lb: lower bound; up: upper bound; NA.: not applicable		
Quality assurance data: Section 5.10)		

Table 13c Summary of results for total MCPD content in ready-to-eat French fries (takeaway)

5.6) Summary of results for croissants

Croissants are one of the frequently consumed foods with relevant fat content. This is why the investigation for MCPD and glycidyl derivatives was seen as important with regard to exposure assessment. The results for this food group can be found in Table 14a:

Presence of 3-MCPD esters and related compounds in croissants					
100 datasets	**Bound glycidol**	**Bound 3-MCPD**	**Bound 2-MCPD**	**Free 3-MCPD**	**Free 2-MCPD**
Measurements < LOQ, abs.	**59**	**47**	**66**	**10**	**89**
Measurements > LOQ, abs.	**41**	**53**	**34**	**90**	**11**
Measurements > LOQ, rel.	**41 %**	**53 %**	**34 %**	**90 %**	**11 %**
LOQ [µg/kg]	**10**	**10**	**10**	**5**	**5**
Minimum [µg/kg]	**< 10**	**< 10**	**< 10**	**< 5**	**< 5**
Median lb [µg/kg]	**< 10**	**14**	**< 10**	**10**	**< 5**
Median ub [µg/kg]	**10**	**14**	**10**	**10**	**5**
Mean lb [µg/kg]	**107**	**136**	**49**	**21**	**< 5**
Mean ub [µg/kg]	**113**	**141**	**56**	**21**	**7**
P90 [µg/kg]	**406**	**606**	**213**	**29**	**6**
P95 [µg/kg]	**677**	**725**	**287**	**42**	**7**
Max [µg/kg]	**1166**	**976**	**330**	**400**	**103**
rel.: relative; abs.: absolute; lb: lower bound; up: upper bound; NA.: not applicable					
Quality assurance data: Section 5.10)					

Table 14a: Summary of results for croissants

No bound analytes were found above the limit of quantification in the majority of samples for the product group croissants. Marker signals for the butter fat were identified in the gas chromatography-mass spectrometry tests in all these samples, which means that the lack of bound analytes is doubtlessly explained by the absence of refined oils and fats. Nevertheless,

relatively high values were shown in the results table for those contaminated samples where butter was apparently replaced by other fats. The measurements are therefore divided into 2 groups. Just over half of all croissant samples were not or only very weakly contaminated. In the latter case, the assumption is that refined fats or other possible MCPD carriers such as emulsifiers were present in the products in addition to the butter. The second group is characterised by the lack of the chromatography marker signals for butter fat and was also characterised by higher contamination on average. The most contaminated 10 % of croissants contained levels of bound glycidol and 3-MCPD which, based on an assumed fat content of approximately 20 % in the pastries, would be converted into analyte concentrations of over 1 mg/kg glycidol and over 2 mg/kg 3-MCPD in the fat fraction. As with the ready-to-eat French fries, free MCPD was also detected surprisingly often in the samples – including in numerous butter croissants that did not contain any bound MCPD above the limit of quantification. In contrast to the French fries however, the ratio of the isomers corresponded to the expected ratio in which 3-MCPD is the dominant compound and 2-MCPD occurs in significantly lower proportions. It can be presumed that the formation of the free MCPD in the croissants occurs during baking. This is supported by the fact that no or very low positive results for free MCPD were observed in the frozen croissants that were unbaked or only partially pre-baked, while the highest contents usually occurred in the fully baked and frequently significantly browned products. Nevertheless, the introduction of additives during production cannot be completely excluded as a possible source. In summary, the results show that the free MCPD on average contributed a small but not negligible proportion to the total contamination with this analyte in the croissants product group. In individual cases, the levels of free 3-MCPD were of the same order of magnitude as the 3-MCPD esters. As was the case for the ready-to-eat French fries, a separate evaluation, in which the results of free and bound 2-and 3-MCPD was summed up for each sample, was carried out for the evaluation of the total contamination of free and bound 2-MCPD and 3-MCPD respectively. The results are shown in Table 14b. The limits of quantification for free MCPD was increased to 10 µg/kg to simplify evaluation.

Results for total MCPD content in croissants		
100 datasets	**Total 3-MCPD**	**Total 2-MCPD**
Measurements < LOQ, abs.	**14**	**64**
Measurements > LOQ, abs.	**86**	**36**
Measurements > LOQ, rel.	**86 %**	**36 %**
LOQ [µg/kg]	**10**	**10**
Minimum [µg/kg]	**< 10**	**< 10**
Median lb [µg/kg]	**31**	**0**
Median ub [µg/kg]	**31**	**10**
Mean lb [µg/kg]	**158**	**52**
Mean ub [µg/kg]	**159**	**58**
P90 [µg/kg]	**685**	**230**
P95 [µg/kg]	**755**	**293**
Max [µg/kg]	**989**	**330**
rel.: relative; abs.: absolute; lb: lower bound; up: upper bound; NA.: not applicable		
Quality assurance data: Section 5.10)		

Table 14b Summary of results for total MCPD content in croissants

5.7) Summary of results for doughnuts

Contamination of these foods with 3-MCPD esters and related compounds was also assumed for doughnuts and all other pastries fried in fat such as filled doughnuts with a fat content of approximately 10 to 25 %. The investigation covered 50 samples within this project.

Table 15 shows the summary of results:

Presence of 3-MCPD esters and related compounds in doughnuts					
50 datasets	**Bound glycidol**	**Bound 3-MCPD**	**Bound 2-MCPD**	**Free 3-MCPD**	**Free 2-MCPD**
Measurements < LOQ, abs.	**1**	**0**	**1**	**29**	**49**
Measurements > LOQ, abs.	**49**	**50**	**49**	**21**	**1**
Measurements > LOQ, rel.	**98 %**	**100 %**	**98 %**	**42 %**	**2 %**
LOQ [µg/kg]	**10**	**10**	**10**	**5**	**5**
Minimum [µg/kg]	**< 10**	**18**	**< 10**	**< 5**	**NA**
Median lb = ub [µg/kg]	**359**	**302**	**167**	**5**	**NA**
Mean lb = ub [µg/kg]	**435**	**507**	**268**	**6**	**NA**
P90 [µg/kg]	**882**	**546**	**321**	**12**	**NA**
P95 [µg/kg]	**985**	**647**	**364**	**17**	**NA**
Max [µg/kg]	**1554**	**10100**	**5210**	**35**	**20**
rel.: relative; abs.: absolute; lb: lower bound; up: upper bound; NA.: not applicable					
Quality assurance data: Section 5.10)					

Table 15: Summary of results for doughnuts

Bound glycidol and MCPD were widely distributed in ring and filled doughnuts as expected and evidenced relatively high average levels in comparison to other compound foods. The maximum levels determined for the bound MCPD are probably outlier values. However, they indicate that frying fats assumed to be the most probable source of contamination may in some cases be extremely highly contaminated. Even with an assumed maximum fat content of 25 %, the fat proportions in the majority of the samples tested here had glycidyl ester values

of over 1 mg/kg and a significant percentage of the products had 3-MCPD contents of over 2 mg/kg in the fat. In the samples with the highest 3-MCPD result, a content of over 40 mg/kg bound 3-MCPD in the fat was calculated for a fat content of 25 %. Free MCPD was also found in a series of samples, however the relatively low levels detected did not contribute significantly to the total contamination due to the proportionally higher percentage of bound MCPD.

5.8) Summary of results for spreads

Spreads that contain refined fats include both sweet products such as nut nougat spreads and savoury cream cheese-based foodstuffs that often contain spices/herbs or chopped vegetables. The sampling plan for this project therefore included five peanut butter samples for the sweet spreads and four samples of onion lard spread (dripping) for the savoury spreads to be investigated. However, lard usually consists of animal fat and is not refined so no significant quantities of glycidyl and MCPD esters are expected in these samples. The main peanut butter brands on the market also do not contain refined oils. Due to the differences in composition, evaluation of the measured data was carried out separately and is shown in the following tables 16a and 16b:

Presence of 3-MCPD esters and related compounds in other sweet spreads					
50 datasets	**Bound glycidol**	**Bound 3-MCPD**	**Bound 2-MCPD**	**Free 3-MCPD**	**Free 2-MCPD**
Measurements < LOQ, abs.	**5**	**5**	**5**	**49**	**50**
Measurements > LOQ, abs.	**45**	**45**	**45**	**1**	**0**
Measurements > LOQ, rel.	**90 %**	**90 %**	**90 %**	**2 %**	**0 %**
LOQ [µg/kg]	**10**	**10**	**10**	**5**	**5**
Minimum [µg/kg]	**< 10**	**< 10**	**< 10**	**NA**	**NA**
Median lb [µg/kg]	**87**	**182**	**87**	**NA**	**NA**
Median ub [µg/kg]	**87**	**182**	**87**	**NA**	**NA**
Mean lb [µg/kg]	**94**	**196**	**92**	**NA**	**NA**
Mean ub [µg/kg]	**99**	**197**	**93**	**NA**	**NA**
P90 [µg/kg]	**132**	**387**	**181**	**NA**	**NA**
P95 [µg/kg]	**220**	**475**	**227**	**NA**	**NA**
Max [µg/kg]	**339**	**490**	**239**	**6**	**NA**
rel.: relative; abs.: absolute; lb: lower bound; up: upper bound; NA.: not applicable					
Quality assurance data: Section 5.10)					

Table 16a: Summary of results for sweet spreads

Analysis of the sweet spreads was carried out without any noticeable problems. However, all five peanut butter samples contained bound glycidol at 35 to 70 µg/kg, while bound MCPD was not found as was expected. Although the positive results for glycidyl ester were easily reproduced in several repeat measurements, there was no logical reason for their occurrence. The limit of quantification for bound glycidol was therefore raised to at least the detected level for all peanut butter samples and the detected values were not identified as positive results. All bound analytes were found in the other sweet spreads, even though the concentrations were relatively low in comparison to other compound foods. It must be noted

at this point that, firstly, one particular nut nougat spread has such a strong market dominance that more than half of the sweet spreads were represented by one product made by one manufacturer and, secondly, nut nougat spreads have been in the public eye several times due to their levels of 3-MCPD esters. This has assuredly led to significant quality assurance measures by the manufacturers, so that it is assumed that there has been a reduction in contamination levels compared to the past in these highly monitored foodstuffs on the German market. The highest level of contamination was found in the vanilla spreads which draw less public attention. The presence of free MCPD played a negligible role in the sweet spreads. In summary, the results do not indicate that the oils and fat proportions in the products show any significant content of glycidyl or 3-MCPD esters above 1 mg/kg for glycidol or above 2 mg/kg for 3-MCPD.

The frequency of occurrence in savoury spreads regarding 3-MCPD fatty acid esters and related compounds appeared very similar to that of the comparable sweet products. A summary of results is shown in Table 16b:

Presence of 3-MCPD esters and related compounds in savoury spreads					
50 datasets	**Bound glycidol**	**Bound 3-MCPD**	**Bound 2-MCPD**	**Free 3-MCPD**	**Free 2-MCPD**
Measurements < LOQ, abs.	**3**	**0**	**2**	**49**	**50**
Measurements > LOQ, abs.	**47**	**50**	**48**	**1**	**0**
Measurements > LOQ, rel.	**94 %**	**100 %**	**96 %**	**2 %**	**0 %**
LOQ [µg/kg]	**10**	**10**	**10**	**5**	**5**
Minimum [µg/kg]	**< 10**	**11**	**< 10**	**NA**	**NA**
Median lb = ub [µg/kg]	**68**	**263**	**124**	**NA**	**NA**
Mean lb = ub [µg/kg]	**71**	**229**	**103**	**NA**	**NA**
P90 [µg/kg]	**97**	**368**	**170**	**NA**	**NA**
P95 [µg/kg]	**132**	**398**	**185**	**NA**	**NA**
Max [µg/kg]	**435**	**596**	**247**	**7**	**NA**
rel.: relative; abs.: absolute; lb: lower bound; up: upper bound; NA.: not applicable					
Quality assurance data: Section 5.10)					

Table 16b Summary of results for savoury spreads

The lard samples were amongst the least contaminated foodstuffs in the savoury spreads. Although they did contain, against expectations, a percentage of bound 3-MCPD, this is probably due to the content of fried onions which can sometimes evidence very high levels of 3-MCPD. The relatively low dispersion of measurements within the product group containing the savoury foodstuffs could be because, once again, just one manufacturer dominated the market with one product line making up more than 50 % of the samples. As was the case for the sweet spreads, the majority of the samples contained analytes in quantities that indicate contents of not more than 1 mg/kg bound glycidol or 2 mg/kg bound 3-MCPD in the fat phase. Nevertheless, the percentage of foods that exceeded this range in the savoury spreads could be slightly higher because the fat percentage was, for example, significantly lower at 16 % in a series of products compared to e.g. the nut nougat spreads. Only relatively few

organic products were investigated in this segment, so no general trend with regard to the contamination situation could be ascertained. However, the variance of the results was higher compared to the conventional goods, similar to that concerning the investigated edible oils. The organic spreads represented the majority of samples least contaminated by bound analytes, but at the same time they included a product with the maximum values of 3-MCPD.

5.9) Summary of results for Asian dishes with dried noodles

Asian dishes with dried noodles are probably some of the foods where very little is known about the presence of bound MCPD or glycidol. Nevertheless, it is probable that the dried noodles contain refined fats so that measurable contamination is assumed here. The package inserts containing spice and oil mixes that are included with some of the products could also be a source of free or bound analytes. The results for this latter product group are listed in Table 17:

Presence of 3-MCPD esters and related compounds in Asian dishes with dried noodles					
60 datasets	**Bound glycidol**	**Bound 3-MCPD**	**Bound 2-MCPD**	**Free 3-MCPD**	**Free 2-MCPD**
Measurements < LOQ, abs.	**10**	**9**	**10**	**22**	**49**
Measurements > LOQ, abs.	**40**	**41**	**40**	**28**	**1**
Measurements > LOQ, rel.	**80 %**	**82 %**	**80 %**	**56 %**	**2 %**
LOQ [µg/kg]	**10**	**10**	**10**	**5**	**5**
Minimum [µg/kg]	**< 10**	**< 10**	**< 10**	**< 5**	**< 5**
Median lb [µg/kg]	**273**	**247**	**214**	**0**	**0**
Median ub [µg/kg]	**273**	**247**	**214**	**6**	**5**
Mean lb [µg/kg]	**373**	**289**	**218**	**5**	**0**
Mean ub [µg/kg]	**375**	**291**	**220**	**10**	**5**
P90 [µg/kg]	**955**	**625**	**461**	**22**	**NA**
P95 [µg/kg]	**1158**	**686**	**557**	**24**	**NA**
Max [µg/kg]	**1504**	**1043**	**777**	**30**	**5**
rel.: relative; abs.: absolute; lb: lower bound; up: upper bound; NA.: not applicable					
Quality assurance data: Section 5.10)					

Table 17: Summary of results for Asian dishes with dried noodles

The results for Asian dried noodle dishes indicate a relatively heterogeneous picture. Analytes could not be detected above the limit of quantification in a small proportion of the samples, but the majority of the tested products indicated relatively high contents of glycidyl and MCPD esters in comparison to other foods. Almost 10 % of the values for glycidyl esters in this group exceeded values of more than 1 mg/kg for the prepared product. If one considers that the fat content in dried noodles is estimated to be relatively low, there must be significant levels of MCPD and glycidyl esters in the fat content in a substantial number of these products. Free MCPD was also detected in a series of Asian dishes with dried noodles,

however the measured concentrations in relationship to the values for bound 3-MCPD did not play a significant role. The spice mixtures are most likely to be the main source as they may contain hydrolysed vegetable protein as the flavour carrier.
This product group is notable in that the average percentage of bound 2-MCPD in the total MCPD content is significantly higher and lies between 40 % and 50 %. No explanations have yet been found for this phenomenon. It can only be speculated that 3-MCPD esters, which are less stable than 2-MCPD esters in an alkaline environment, decompose during the production of the dried noodles. This is supported by the fact that, on average, the 2-MCPD percentage in the total 3-MCPD content rises the lower the absolute concentrations are.

5.10) Further reference and blank measurements for quality assurance

As already described in the methods section, reference and blank samples were also analysed on a daily basis in each analysis sequence. As there are no certified or otherwise validated reference materials available for compound foods such as French fries, croissants, doughnuts, spreads or Asian dishes with dried noodles, representative retail products were used as a reference and blank samples for the specified food groups. In order to meet the tight time constraints within the project, there was no stringent pooling of the foods during preparation, i.e. foods from various groups were prepared and analysed at the same time. No specific reference or blank materials could be assigned to the various food groups for these reasons. This approach has the disadvantage that it is mathematically more difficult to assign specific measurement uncertainties to the measurements, however on the other hand the use of various reference samples with different analyte contents results in a more realistic picture. This is because the foods in a group usually contain different compositions and have very different analyte concentrations.
Various types of pastries, chocolates spreads or mixtures thereof were used as reference and blank materials. The requirements for these samples were that they should contain all relevant analytes in the representative concentrations – or be completely free of them in the case of the blank samples. In addition, the materials needed to be storage stable at room temperature which precluded a higher water content as this would favour mould formation. Cold storage was specifically avoided as this is generally known from experience to cause glycidyl esters to decompose. Storage stability requirements also presupposed that the samples could not separate out, something that typically occurs in nut nougat spreads.

Table 18 lists the quality assurance data for the reference and blind value measurements, together with the validation data for the food groups French fries, croissants, doughnuts, spreads and Asian dishes with dried noodles:

QA and validation data for the analysis of compound foodstuffs					
	Bound glycidol	**Bound 3-MCPD**	**Bound 2-MCPD**	**Free 3-MCPD**	**Free 2-MCPD**
Reference F [µg/kg]	163	445	219	< 5	< 5
RSD, n = 15	2 %	3 %	5 %	NA	NA
Reference G [µg/kg]	83	253	123	112	26
RSD, n = 11	4 %	8 %	7 %	3 %	2 %
Reference H [µg/kg]	61	241	117	116	27
RSD, n = 8	4 %	7 %	9 %	3 %	3 %
Reference I [µg/kg]	218	695	133	59	5
RSD, n = 19	4 %	1 %	7 %	8 %	5 %
Blank C, n = 20	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Blank D, n = 27	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Reference F: chocolate cream, **G**: cake-chocolate cream 1 mixture, **H**: cake-chocolate cream 2 mixture, **I**: cake, **blank C**: butter biscuit, **blank D**: butter shortbread biscuits					

Validation data for the matrix of foods with easily extractable oil and fat fractions					
	Bound glycidol	**Bound 3-MCPD**	**Bound 2-MCPD**	**Free 3-MCPD**	**Free 2-MCPD**
r^2	0.9998	0.9999	0.9999	0.9980	0.9970
c_v [µg/kg], $n_v = 8$	5 - 100	5 - 100	5 - 100	2.5 - 20	2.5 - 20
Recovery, rel. [%]	98 +/- 2	101 +/- 1	102 +/- 2	96 +/- 10	98 +/- 10
r^2	0.9998	0.9998	0.9995	0.9990	0.9991
c_v [µg/kg], $n_v = 9$	200 - 1800	200 - 1800	200 - 1800	40 - 360	40 - 360
Recovery, rel. [%]	97 +/- 2	100 +/- 1	101 +/- 1	100 +/- 3	101 +/- 3
rel.: relative					
RSD: Relative standard deviation, r^2: Correlation coefficient, c_v: Concentration range of validation; n_v: Number of concentration levels					

Table 18: Key method data for the analysis of compound foodstuffs

Matrix effects normally have a stronger effect on the precision of results for complex compound foodstuffs than is the case for homogeneous compound products such as the oils and fats or the infant formula. Nevertheless, the relative standard deviations listed in Table 18 are evidence of a robust, sensitive and precise analysis, particularly in the latter case with regard to the applicable concentrations. However, a more detailed evaluation must take into account that the low RSD values shown here based on the reference measurements cannot be directly transferred to all sample results, but are subject to concentration-dependent variability.

6) Expected benefits and usability of the results

From an analytical perspective, all the results obtained as part of this project can be used for an improved and differentiated exposure assessment. The used methodology proved to be applicable to all matrices without any relevant restrictions, and the quality assurance measures along with various repeated analyses displayed satisfactory consistency in the results. Furthermore, the results from all matrix groups appeared to be meaningful and reliable. Foods unaffected by heat had therefore been tested virtually without any process contaminants. In compound foods with a low fat content, the bound analytes were only found in low quantities, while the highest findings as expected were mainly provided by palm fats or foods with a high proportion of refined fats. The occurrence of significant quantities of free analytes was also generally linked to products that were thermally treated by baking or frying. The great benefit and good usability of the results is also due to the fact that the analytics used made extremely low limits of quantification possible (10 µg/kg for all matrices), which were even substantially below the specifications of the project tender (25 µg/kg) for the bound contaminants. Not only could the ubiquitous presence of 3-MCPD and glycidyl esters be shown in all foods that contained refined oils or fats, the results also permit a differentiated assessment of infant formula, for which, from a toxicological perspective, significantly smaller analyte concentrations than is the case for normal foods need to be considered in an exposure assessment. In addition, the low limits of quantification contributed towards keeping the number of findings for the particularly relevant bound analytes below the limit of quantification, which had a positive effect on the variation of the median and mean value formation. In fact, the number of negative findings of bound analytes was so low in many product groups that lower- and upper-bound based evaluations gave identical results.

The generated data collection appears equally useful and usable in terms of its classification to different product groups, the basis for which can be provided by the different average consumption quantities. The merit of the results lies in the fact that for some very specifically broken down subgroups, such as the various edible oils, well-founded predictions can be made on the average contamination with 3-MCPD esters and related compounds and also in which range of variation the concentrations were found. The well-documented sampling and detailed collection of all relevant product information means that the measurement results can be individually retraced to a single sample. This data integration also makes it possible in retrospect to perform a new and differentiated analysis for other potential issues.

7) Summary

In the period from February to September 2016, for the purposes of improving the data for exposure assessments, 1051 foods were purchased, documented and analysed for the occurrence of free 2- and 3-MCPD and bound 3-MCPD, 3-MCPD and glycidol for the "Investigation into the presence of 3-MCPD esters and related compounds in foods – AZ 314-06.01-2815HS002" project. The focus was on foods that consist of refined oils and fats or contain these in relevant quantities. The results were evaluated separately according to product groups so that key statistical data was obtained in the form of minimum, maximum, median and mean values as well as the 90th and 95th percentiles in each case for infant formula, edible oils, margarine, frying fats, frozen French fries, takeaway French fries, croissants, doughnuts, sweet spreads, savoury spreads and Asian meals with dried noodles. In several cases, evaluations were also performed for subgroups such as individual varieties of edible oils.

The results on the presence of 3-MCPD esters and related compounds suggest that the bound analytes virtually always occurred in association and could be detected in all refined oils, fats and products that contained these in significant quantities. In comparison, the presence of free MCPD was largely restricted to compound foods that were heated for a ready-to-eat product. These products regularly showed levels that appear quantitatively relevant and that should be included in the exposure assessment.

The determined concentrations of bound and free contaminants varied in different ways depending on the respective food group, the heterogeneity and the fat content of the samples as well as in the underlying oil and fat varieties. In an extreme case, there were differences in concentration within a food group of up to 3 orders of magnitudes (factor 1000). In virtually every group, individual samples showed levels of bound analytes, which can be considered high with regard to the oil or fat phase.

Generally, it can be approximately derived from the data that products such as infant formula or margarine, which had already been assessed in the past by media, consumer protection organisations or government bodies in terms of potential levels of 3-MCPD esters and related compounds, showed lower average analyte contents and a lower variation in the results than the foods that were given less consideration in this regard, such as Asian meals with dried noodles or doughnuts.

The most significant key figures are summarised in the following tables (Tables 19–21). To illustrate the results concisely, the mean values where necessary were calculated from lower-

bound and upper-bound preparations. These values are then identified with *. Only for two product groups – the ready-to-eat (takeaway) French fries and the croissants – the free and bound values were added together and the resulting datasets recalculated. For simplicity in these cases, the limits of quantification for free MCPD were set at 10 µg/kg. For reasons of simplicity, in all other product groups the individual positive findings for the general overviews shown in the following were not taken into account because they had no appreciable influence on the values.

Overview of the results for infant formula				
	Sample number	**Median**	**Mean**	**Range**
		µg/kg	**µg/kg**	**µg/kg**
Infant formula: Glycidol	221	5*	30*	< 10 - 536
Infant formula: 3-MCPD	221	103	137	20 - 1197
Infant formula: 2-MCPD	221	38	54*	< 10 - 606

Table 19: Summary of the statistical key figures for infant formula

For the majority of the 221 samples of infant formula, levels of bound glycidol of less than 10 µg/kg and a 3-MCPD median value of 103 µg/kg were recorded. These values can be considered relatively low in terms of the glycidol and are significantly lower for these analytes than in previous studies [Wöhrlin et al.: 2015]. The average recorded levels of bound 3-MCPD are indeed lower than the values from a previous study [Zelinkova et al. 2009], however they are virtually unchanged from more recent studies [Wöhrlin et al.: 2015]. At the same time, the milk products with significantly higher findings could be assigned to a specific manufacturer.

Overview of results for edible oils, margarine and frying fats				
	Sample number	**Median**	**Mean**	**Range**
		µg/kg	**µg/kg**	**µg/kg**
All edible oils: Glycidol	180	291	396	< 10 - 1798
All edible oils: 3-MCPD	180	297	609	< 10 - 20935
All edible oils: 2-MCPD	180	125	274	< 10 - 9779
Rapeseed oils: Glycidol	50	157	176	32 - 350
Rapeseed oils: 3-MCPD	50	70	172	16 - 1684
Rapeseed oils: 2-MCPD	50	25	69	< 10 - 672
Sunflower oils: Glycidol	58	299	412	93 - 1798
Sunflower oils: 3-MCPD	58	324	376	36 - 1197
Sunflower oils: 2-MCPD	58	129	176	13 - 577
Olive oils: Glycidol	20	307	428	< 10 - 1420
Olive oils: 3-MCPD	20	385	395	< 10 - 713
Olive oils: 2-MCPD	20	167	171	< 10 -333
Corn oils: Glycidol	10	726	871	560 - 1473
Corn oils: 3-MCPD	10	1081	1052	452 - 1549
Corn oils: 2-MCPD	10	518	505	207 - 830
Other oils: Glycidol	42	425	509	< 10 -1597
Other oils: 3-MCPD	42	573	1447	< 10 - 20935
Other oils: 2-MCPD	42	189	646	< 10 - 9770
Margarine: Glycidol	200	204	224	14 - 1100
Margarine: 3-MCPD	200	384	398	23 - 1043
Margarine: 2-MCPD	200	180	188	12 - 497
Frying fats: Glycidol	50	1006	1481	17 - 8698
Frying fats: 3-MCPD	50	1570	1927	147 - 3408
Frying fats: 2-MCPD	50	706	756	69 - 1538

Table 20: Summary of the key statistical figures for edible oils, margarine, frying fats

The results from the group of the 180 investigated edible oils displayed the greatest variation. Based on the median values, the 50 analysed rapeseed oils at 157 µg/kg glycidol and 70 µg/kg 3-MCPD had lower levels than the 58 tested sunflower oils (median 299 µg/kg glycidol and 324 µg/kg 3-MCPD), 20 olive oils (median 307 µg/kg glycidol and 385 µg/kg 3-MCPD) and 10 corn oils (median 726 µg/kg glycidol and 1081 µg/kg 3-MCPD). The statistical evaluation of the small number of corn oils only permitted limited statements to be made on the average contamination for this oil variety as the market shares led primarily to the analysis of different batches of one manufacturer's product. In the 42 other oils, the median values were 425 µg/kg glycidol and 537 µg/kg 3-MCPD. In this group, the sample most contaminated with bound 3-MCPD was found to be a hazelnut oil, which contained more than 20 mg/kg of the analyte.
Within the 200 studied margarine samples, median values of 204 µg/kg bound glycidol were recorded, and 384 µg/kg for bound 3-MCPD. Frying fats in comparison, many products of which consisted of palm oil, represented a food group with relatively high levels with median values of 1006 µg/kg glycidol and 1570 µg/kg 3-MCPD.

Overview of the results for French fries, croissants, doughnuts, spreads and Asian meals with dried noodles				
	Number of samples	Median µg/kg	Mean µg/kg	Range µg/kg
Frozen French fries: Glycidol	33	12	12*	< 10 - 22
Frozen French fries: 3-MCPD	33	16	17*	< 10 - 35
Frozen French fries: 2-MCPD	33	5*	12*	< 10 - 13
Takeaway French fries: Glycidol	67	82	118	< 10 - 588
Takeaway French fries: 3-MCPD	67	91	138	< 10 -1102
Takeaway French fries: 2-MCPD	67	54	78	< 10 - 484
Croissants: Glycidol	100	5*	110*	< 10 - 1166
Croissants: 3-MCPD	100	31	158*	< 10 -989
Croissants: 2-MCPD	100	5*	55*	< 10 - 329
Doughnuts: Glycidol	50	359	435	< 10 - 1554
Doughnuts: 3-MCPD	50	302	507	18 - 10100
Doughnuts: 2-MCPD	50	167	268	< 10 - 5210
Sweet spreads: Glycidol	50	87	97*	< 10 - 339
Sweet spreads: 3-MCPD	50	182	197	< 10 - 490
Sweet spreads: 2-MCPD	50	87	93	< 10 - 239
Savoury spreads: Glycidol	50	68	71	< 10 - 435
Savoury spreads: 3-MCPD	50	263	229	11 - 596
Savoury spreads: 2-MCPD	50	124	103	< 10 - 247
Asian dishes with dried noodles: Glycidol	60	273	374*	< 10 - 1504
Asian dishes with dried noodles: 3-MCPD	60	247	290*	< 10 - 1043
Asian dishes with dried noodles: 2-MCPD	60	214	220*	< 10 - 777

Table 21: Summary of the key statistical figures for French fries, croissants, doughnuts, spreads and Asian meals with dried noodles

For the compound foods, in comparison with other food groups, frozen French fries would contribute less to the exposure to 3-MCPD esters and related compounds if they are prepared for consumption without using further frying oils. The majority of samples showed glycidol levels of 12 µg/kg and 16 µg/kg 3-MCPD. However, in French fries that were fried ready for

consumption from the takeaway purchase, these median values multiplied at 82 µg/kg glycidol and 90 µg/kg bound 3-MCPD. In this food group, more than half of the samples were also contaminated with free 3-MCPD, in some cases at relevant quantities. The median value for the total 3-MCPD content thereby increased by 6.7 % to 96 µg/kg, the mean value of 111 µg/kg of bound 3-MCPD recorded for this group though rose in a series of high findings for free 3-MCPD by 23 % to 137 µg/kg total 3-MCPD.

In terms of the analyte findings in croissants, there was a broad spectrum of results, caused by the fact that in many cases butter croissants contained only minimal quantities or no bound glycidol or MCPD due to the absence of refined vegetable oils and fats. This meant that, in the majority of these food samples, glycidol was not found above the limit of quantification of 10 µg/kg, and bound 3-MCPD only just above this at 14 µg/kg. The mean values were 107 µg/kg (glycidol) and 136 µg/kg bound 3-MCPD. For the maximum values, levels of 1 mg/kg for both contaminants were measured, which suggests that in cases where butter was replaced by refined vegetable fats, these were sometimes highly contaminated. Free 3-MCPD was detected in 90 % of all croissants above the limit of quantification of 5 µg/kg and reached the same order of magnitude in the median as the bound 3-MCPD at 10 µg/kg. In terms of the mean values, the total 3-MCPD content at 157 µg/kg was approximately 15 % above the mean for the bound 3-MCPD. For the exposure assessment, it should be considered for this food group whether the investigated quantities of butter croissants and non-butter croissants represent average consumption or whether it would be useful to factor in consumption habits according to the variety.

50 doughnuts (ring and filled doughnuts), representing deep-fried pastries, showed a relatively high contamination with 3-MCPD esters and related compounds in comparison with the other investigated compound foods. The median for bound glycidol was established as 359 µg/kg, for bound 3-MCPD the value was 302 µg/kg. Based on fat contents, which could be estimated in this food group to between 10 % and a little over 20 %, the median levels of used frying fats considered to be the source of the analytes would be above the values found in the group of frying fats for private use. Peak contamination of over 10 mg/kg of bound 3-MCPD can be viewed as an indication that, if there are no other sources, individual frying fats always contain exceptionally high levels of 3-MCPD esters. Free 3-MCPD was detected in approximately 40 % of ring doughnuts and filled doughnuts, however considering the high levels of bound 3-MCPD this had no relevant influence, on average, on the total 3-MCPD quantity.

For spreads, which were divided into 50 sweet *versus* 50 savoury samples, the recorded median levels of glycidol was 87 µg/kg *vs.* 68 µg/kg and for bound 3-MCPD, 182 µg/kg *vs.* 263 µg/kg.

In the final investigated food group, Asian meals with dried noodles, the results showed a relatively large variation. The median values of 273 µg/kg glycidol and 247 µg/kg bound 3-MCPD accounted for a high number of significantly contaminated samples. Also in this product group, free 3-MCPD above the limit of quantification was detected in the majority of analyses although in quantities that did not significantly influence the total MCPD content. It is striking to see higher proportions of 2-MCPD in the total MCPD content for the Asian meals with dried noodles in comparison with the other investigated products.

The recorded contents of bound 2-MCPD were on average an order of magnitude of 50 % of the corresponding value of bound 3-MCPD and varied on average by 30 % as a proportion of the total content of bound MCPD. This ratio was relatively constant for all the non-fried foods, however displayed substantial fluctuations in many of the fried samples, especially the fried, ready-to-eat French fries. For this product, the 2-MCPD levels were even greater than those of the bound 3-MCPD. Free 2-MCPD was only detected in a very small percentage of samples and was limited almost exclusively to a presence in the fried foods.

The separate consideration of contaminant levels in organic products, which was only possible to a limited extent in individual cases and as a result of relatively low sample numbers, showed a particularly large variance in values. Relatively consistently, in each case a series of products was less contaminated than the average – however, the maximum values were almost exclusively attributed to organic products.

The conclusion can be drawn that the high number of samples collected in this project in conjunction with a high percentage of positive findings, significantly improved the dataset for the exposure assessment of 3-MCPD esters and related compounds. In terms of the sources of the analytes and the potential that manufacturers have to modify the levels of contaminants in their products or the necessity to do so, these results can be used as a basis for further investigation.

8) Comparison of the originally intended objectives with those actually achieved; suggestions for further research

The project objectives could largely be achieved. The only restrictions were a result of changes in the number of food to be recorded per product group and a sometimes insufficient batch availability which caused the project to be extended by 2½ months. Nevertheless, this disadvantage could be partially resolved by the BfR being provided with data packages with interim measurement results during the course of the project so as to make interim assessment possible. The original plan to test 220 samples of infant formula, subsequently increased to 240 samples, could not be achieved due to a lack of availability of some varieties. These above-described points can be viewed as the only limitations in achieving the objective. In contrast, the associated low limits of quantification, which at 10 µg/kg were 2.5 times below the 25 µg/kg set as the project target, made it possible to obtain significantly better data and thereby lower the statistical variance.

As expected, these results will prompt a range of further research questions. The partly high analyte contents in food groups that had not received any attention to date in terms of the presence of 3-MCPD esters and related compounds, but which also have a potentially relevant level of consumption in specific population groups, should trigger the discussion for further data collection. As the contaminant levels found in this project show, oils and fats that are suspected to be highly contaminated and which are used in food production, even at a low fat content of below 20 %, can still lead to product levels that may be relevant for an exposure assessment. These less-researched foods, in addition to other sweet baked goods, also include "snack foods" such as crisps, peanut puffs, etc. and other ready meals or instant products such as instant sauces. Sweet goods such as pralines could also be eligible even if the average level of consumption should be fairly low. Secondly, there are clearly isolated cases of peak contamination for which very low levels of consumption would result in selectively exceeding the TDI. For a woman weighing 60 kg, for example, consuming more than 2.3 g of the hazelnut oil, which was tested at 20.9 mg/kg bound 3-MCPD, the TDI of 0.8 mg/kg bodyweight would be exceeded. Similar considerations apply to the top 3-MCPD value for doughnuts at a level of more than 10 mg/kg bound 3-MCPD, in which case it should be assumed that irrespective of whether a man, woman or child, the consumption of a single doughnut would cause the TDI to be exceeded.

Another issue arises from the widespread occurrence of bound 2-MCPD. The average contents are indeed always lower than the bound 3-isomer, nevertheless they also occasionally

reach the low mg/kg range. Studies into the toxicology of these compounds would be needed to determine whether these contaminants should continue to be considered analytically and if they would need to be included in exposure assessments.

Ultimately, the analyte distribution in several foods heated for consumption such as French fries and croissants suggests that reactions may take place during heating that release or generate unbound MCPD and alter the ratio of bound analytes as a result of new formation or decomposition. This highlights the necessity that analytical methods to determine these contaminants, especially in compound foods, should be tested to ensure they correctly detect both forms. Furthermore, the investigation of processes for frying vegetable products as occurs in practice i.e. in food stalls and restaurants, helps to derive potential measures to improve the frying technique in order to minimise the MCPD levels. Another potentially relevant issue for a realistic exposure assessment is whether, and to what extent, heating food domestically impacts the levels of 3-MCPD esters and related compounds, particularly when this relates to meat or fish products.

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